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PATENT APPLICATION
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(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	600-1-257 CIP
First Inventor or Application Identifier	James A. Bibb
Title	METHODS OF IDENTIFYING AGENTS ...
Express Mail Label No.	EL684491492US

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.**ADDRESS TO:**
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1. ☐ * Fee Transmittal Form (e.g., PTO/SB/17)
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(preferred arrangement set forth below)
 - Descriptive title of the Invention
 - Cross References to Related Applications
 - Statement Regarding Fed sponsored R & D
 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
 - Detailed Description
 - Claim(s)
 - Abstract of the Disclosure
3. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets **12**]
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Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

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7. ☐ Assignment Papers (cover sheet & document(s))
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|---------------------------------------|-------------------------------------|--|--|

Prior application information: Examiner **R. Shukla**Group / Art Unit: **1632**

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Applicant or Patentee: James A. Bibb and Paul Greengard

Application or Patent No.: 09/419,379

Filed or Issued: October 15, 1999

For: METHODS OF IDENTIFYING AGENTS THAT REGULATE PHOSPHORYLATION/DEPHOSPHORYLATION IN DOPAMINE SIGNALING

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 C.F.R. §§ 1.9(f) AND 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION THE ROCKEFELLER UNIVERSITY

ADDRESS OF ORGANIZATION 1230 York Avenue
New York, New York 10021-6399

TYPE OF ORGANIZATION

- ☒ University or other institution of higher education
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☐ Nonprofit scientific or educational under statute of state of The United States of America
(Name of state _____)
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I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. § 1.9(e) for purposes of paying reduced fees under Sections 41(a) and 41(b) of Title 35, United States Code, with regard to the invention entitled METHODS OF IDENTIFYING AGENTS THAT REGULATE PHOSPHORYLATION/DEPHOSPHORYLATION IN DOPAMINE SIGNALING by inventor(s) JAMES A. BIBB and PAUL GREENGARD described in

- ☐ the specification filed herewith
☒ Application No. 09/419,379, filed October 15, 1999
☐ Patent No. _____, issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

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individual inventor under 37 C.F.R. § 1.9(c), or by any concern that would not qualify as either a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING William H. Griesar

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SIGNATURE



DATE 11/22/99

CROSS-REFERENCE TO RELATED APPLICATIONS

GOVERNMENTAL SUPPORT

15 FIELD OF THE INVENTION

The present invention discloses that DARPP-32 can be phosphorylated at a specific threonine residue by the cyclin dependent kinase 5 (Cdk5). The phosphorylation is shown to significantly alter the properties of DARPP-32. The present invention provides the phosphorylated protein, fragments comprising the phosphorylated
20 threonine residue, and antibodies to the phosphorylated protein. The present invention further provides methods of treating dopamine dysfunction, as well as methods of identifying additional agents which modulate the phosphorylation of DARPP-32 by Cdk5 for such treatments.

BACKGROUND OF THE INVENTION

Dopamine plays an important role as a neurotransmitter in the mammalian nervous system. Indeed, the selective dysregulation of dopaminergic neural transmission has been indicated in a number of neurological disorders including schizophrenia,
 5 Parkinson's disease, Huntington's disease, symptoms of attention deficit hyperactivity disorder, Tourette's syndrome, and drug abuse. Virtually all current anti-schizophrenic drugs act as antagonists at a major subclass of dopamine receptors, but are not completely effective and produce undesirable side effects. In addition, cocaine, amphetamines, opiates, nicotine and alcohol have all been shown to be
 10 capable of modifying dopaminergic transmission. Therefore, dopaminergic transmission is an important factor in maintaining the mental health of an individual.

A major target for midbrain dopaminergic neurons is the neostriatum. About 95% of all neostriatal neurons have a similar morphology and are referred to as medium-sized spiny neurons. Dopamine- and cyclic AMP (cAMP)-Regulated PhosphoProtein
 15 (DARPP-32) is a 32 kilodalton cytosolic protein that is selectively enriched in medium-sized spiny neurons in neostriatum [Ouimet *et al.*, *Neurosci* **4**:114-124. (1984); Walaas and Greengard, *J Neurosci* **4**: 84-98 (1984)]. The sequence of Human DARPP-32 has been determined [Brene *et al.*, *J. Neuroscience*, **14**:985-998 (1994); GenBank Accession: AAB30129.1] and a knockout mouse lacking DARPP-32 has
 20 been constructed [U.S. Patent No:5,777,195, Issued July 7, 1998; Fienberg *et al.*, *Science* **281**:838-842 (1998), the contents of each are hereby incorporated by reference herein, in their entireties].

DARPP-32 is phosphorylated by cAMP-dependent protein kinase (PKA) on a single threonine residue, the thirty-fourth amino acid in the sequence, *i.e.*, Thr34 which
 25 results in the conversion of DARPP-32 into a potent inhibitor of protein phosphatase-1 (PP1) [Hemmings *et al.*, *Nature* **310**: 503-505 (1984)]. DARPP-32 can be dephosphorylated at Thr34 *in vitro* by the calcium/calmodulin-dependent protein

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phosphatase, calcineurin [King *et al.*, *J Biol Chem* **259**:8080-8083. (1984)].

Dephosphorylation of Thr34 of DARPP-32 removes its inhibitory effect on PP1.

Dopamine has been shown to stimulate the phosphorylation of DARPP-32 in the neostriatum by activation of a biochemical cascade involving stimulation of D1
 5 receptors, activation of adenylyl cyclase, increased cAMP formation and increased activity of PKA [Walaas and Greengard, *J Neurosci* **4**:84-98 (1984)]. The selective enrichment of DARPP-32 in dopaminoceptive neurons and its regulation by dopamine strongly indicate that DARPP-32, through regulating protein phosphatase-1 activity, plays a key role in mediating the effects of dopamine on these cells. Indeed, in the
 10 brain the chain of events has been described as the DARPP-32/Protein Phosphatase-1 cascade [Greengard *et al.*, *Neuron*, **23**:435-447 (1999)].

The control of protein phosphatase-1 activity by DARPP-32 is likely to have a significant role in the regulation of neuronal excitability. For instance, in neostriatum, dopamine-mediated effects on the function of calcium channels [Surmeier *et al.*,
 15 *Neuron* **14**:385-397 (1995)], voltage-dependent sodium channels [Surmeier *et al.*, *Proc. Natl. Acad. Sci., USA* **89**:10178-10182 (1992); Schiffman *et al.*, *Am J Physiol* **483**:95-107 (1994)] and Na⁺,K⁺-ATPase [Aperia *et al.*, *Proc Natl Acad Sci, USA* **88**:2798-2801 (1991)] are all regulated directly or indirectly by protein phosphatase-1.

Medium-sized spiny neurons of the neostriatum and nucleus accumbens receive
 20 dopaminergic input from cell bodies in the midbrain [Anden *et al.*, *Life Science* **3**:523-530 (1964); Poirier and Sourkes, *Brain* **88**:181-192 (1965); Swanson, *Brain Res Bull* **9**: 321-353 (1982)]. To date, five dopamine receptor subtypes have been identified which constitute two major subclasses, a D1 subfamily (D1 and D5 subtypes) and a D2 subfamily (D2, D3 and D4 subtypes) [Sibley and Monsma, *Trends*
 25 *in Pharmacol Sci* **13**:61-69. (1992)]. D1 and D2 dopamine receptors are abundantly expressed on cell bodies and dendritic processes of medium spiny neurons [Levey *et al.*, *Proc Natl Acad Sci, USA* **90**:8861-8865 (1993)]. Messenger RNAs coding for each of the other dopamine receptor subtypes (i.e., D3, D4, and D5) have been

isolated from individual neostriatal neurons [Surmeier *et al.*, *J Neurosci* **16**:6579-91 (1996)], but whether these receptor proteins are expressed in medium spiny neurons and how they functionally interact with D1 and D2 receptors is still unclear.

There is considerable evidence for either synergistic or opposing interactions of D1-like and D2-like dopamine receptors at the biochemical, physiological, and behavioral level [see Jackson and Westlind-Danielsson, *Pharmac Ther* **64**:291-370 (1994) for review]. Biochemically, D1 and D2 receptors have opposing actions on the activity of adenylyl cyclase in neostriatal neurons; whereas activation of D1 receptors increases cAMP formation by adenylyl cyclase, D2 receptors inhibit adenylyl cyclase activity [Stoof and Kebabian, *Nature* **294**: 366-368 (1981)]. Studies have shown that D2-like dopamine receptors *via* interactions with specific G-proteins, can be coupled to multiple effector systems, including calcium channels, potassium channels and phospholipase C [for review, see Huff, *Cell Signal* **8**: 453-459 (1996)]. For example, Yan *et al.* [*Soc. Neurosci. Abst.* **26**:1088 (1996)] have shown that D2 receptors on neostriatal neurons negatively couple to calcium channels through a $G_{i/o}$ class protein. In addition, activation of D2 receptors apparently decreases sodium currents in medium spiny neostriatal neurons through a membrane-delimited pathway and increases these currents through a soluble second messenger pathway (presumably involving inhibition of adenylyl cyclase) [Surmeier *et al.*, *Proc. Natl. Acad. Sci., USA* **89**:10178-10182. (1992)].

Heretofore, there has been no particular link between DARPP-32 and Cdk5, a member of the cyclin-dependent kinases (cdks) [See generally, Sherr, *Cell* **79**:551-555 (1994) and Sherr, *Cell* **73**:1059-1065 (1993)]. However, Cdk5 [also known as neuronal cyclin-dependent-like protein (Nclk) and tau protein kinase II (TPKII)] has been reported to function in cortical lamination, neurite outgrowth, neuronal plasticity, ischemia, apoptosis, myogenesis and in estrogen signal transduction. This kinase has also been shown to be involved in the hyper-phosphorylation of neurofilaments, which form neurofibrillary tangles observed in a number of neurodegenerative diseases.

However, whereas active cyclin dependent kinases consist of a positive regulatory subunit (the cyclin) and a catalytic subunit (the cyclin dependent kinase) Cdk5

Cyclin dependent kinases play an important role during the cellular replication cycle with the regulation of the human cell cycle requiring the periodic formation, activation, and inactivation of protein kinase complexes that consist of a cyclin subunit and a cdk subunit. Indeed, there has been significant interest in cdks in regard to cancer treatment due to the role of cdks in cell division. In contrast, in adults, Cdk5 is not only most highly expressed in the brain, it is also expressed throughout the brain, and furthermore is only active in the brain. Since brain cells are for the most part post-mitotic, *i.e.*, they no longer divide, Cdk5 also is an atypical member of the cyclin-dependent kinase family because it appears to have a role that is independent of cell division.

Heretofore, most of the drugs that are used to treat dopamine-related disorders function at the extracellular surfaces as either D1 receptor agonists or D2 receptor antagonists. These compounds often have a limited period of efficacy and produce unwanted side-effects. Many of these unwanted side-effects are the result of the lack of specificity of the drug for its target. Therefore, there is a need to provide new drugs assays which can be used to develop novel drugs that can be used to treat dopamine-related disorders. Such novel drugs would have greater specificity than those currently used and therefore, would be less likely to have unwanted side-effects. Furthermore, there is a need to develop treatments for diseases/conditions which are

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SUMMARY OF THE INVENTION

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acid substitution, and the threonine residue is the seventy-fifth (75) amino acid residue of the amino acid sequence.

The present invention also provides phosphorylated fragments of a DARPP-32 protein in which the fragments comprise a phosphorylated threonine residue, that when
 5 dephosphorylated, can be re-phosphorylated by Cdk5. In a particular embodiment the phosphorylated mammalian DARPP-32 protein fragment is a fragment of a DARPP-32 protein having the amino acid sequence of SEQ ID NO:1, and the threonine residue is the seventy-fifth (75) amino acid residue of the amino acid sequence of SEQ ID NO:1. In a related embodiment the phosphorylated mammalian DARPP-32
 10 protein fragment is a fragment of a DARPP-32 protein has the amino acid sequence of SEQ ID NO:1 having a conservative amino acid substitution, and the threonine residue is the seventy-fifth (75) amino acid residue of the amino acid sequence.

The present invention also provides fusion and/or chimeric peptides and proteins comprising the fragments of phosphoThr75 DARPP-32 protein or the full-length
 15 phosphoThr75 DARPP-32 protein.

The present invention further provides phosphorylation state-specific antibodies that have specificity for a phosphorylated mammalian DARPP-32 protein comprising a phosphorylated threonine residue that can be reversibly phosphorylated and dephosphorylated, and which when that threonine residue is dephosphorylated, the
 20 threonine can be re-phosphorylated by Cdk5. In a preferred embodiment the Thr75-phosphorylated DARPP-32 has the amino acid sequence of SEQ ID NO:1. In a related embodiment the Thr75-phosphorylated DARPP-32 has the amino acid sequence of SEQ ID NO:1 having a conservative amino acid substitution. In a particular embodiment the phosphorylation state-specific antibody is a polyclonal
 25 antibody. In another embodiment the phosphorylation state-specific antibody is a monoclonal antibody. In still another embodiment the phosphorylation state-specific antibody is a chimeric antibody.

The present invention also provides methods of identifying agents (or drugs) that can modulate the phosphorylation state of Thr75 DARPP-32. Such agents are preferably small organic molecules. One such method comprises contacting a potential agent with Cdk5 and DARPP-32 and determining the amount phosphorylation of

- 5 DARPP-32. A potential agent is identified as an agent that can modulate the phosphorylation state of Thr75 DARPP-32 if the amount of phosphorylation of DARPP-32 determined is significantly changed in the presence of the potential agent relative to in the absence of the agent. When the amount of phosphorylation is increased the agent is identified as an agonist of Thr75 DARPP-32 phosphorylation, 10 whereas when the amount of phosphorylation is decreased the agent is identified as an antagonist of Thr75 DARPP-32 phosphorylation

In an alternative embodiment, the rate of phosphorylation of Thr75 DARPP-32 is determined either alone or with the determination of the amount of phosphorylation.

- In a particular embodiment, an analog of Cdk5 is employed either as the sole kinase or 15 along with Cdk5. In another embodiment the substrate of the analog of Cdk5 is a Cdk5 phosphorylatable fragment of DARPP-32.

- The above methods can further comprise contacting the agent with an alternative protein kinase such as a MAP kinase and a substrate for that alternative kinase. The amount (and/or rate) of phosphorylation of the substrate by the alternative kinase is 20 then determined. The alternative kinase preferably is known not to phosphorylate DARPP-32 on Threonine-75. An agent is identified as an agent that can modulate the phosphorylation state of Thr75 DARPP-32 if the amount (and/or rate) of phosphorylation of the substrate for the alternative kinase is not significantly changed in the presence of the agent relative to in the absence of the agent. In a preferred 25 embodiment, the alternative protein kinase is glycogen synthase kinase-3 β . More preferably the alternative protein kinase is another cyclin-dependent kinase such as cdk1/cyclin B.

- The present invention also provides *in vivo* methods of identifying agents (or drugs) that can modulate the phosphorylation state of Thr75 DARPP-32. Such methods can be employed alone or in conjunction with *in vitro* and *in situ* methods as exemplified herein. One such method comprises administering the agent to a non-human
- 5 mammal, preferably along with a dopamine D1 receptor agonist. The amount (and/or rate) of phosphorylation of the PKA substrate is then determined. Since the administration of the dopamine D1 receptor agonist in the absence of the agent results in an increase in the phosphorylation state of a PKA substrate an agent is identified as capable of modulating the phosphorylation state of Thr75 DARPP-32 when the
- 10 amount (and/or rate) of phosphorylation of the substrate is significantly decreased in the presence of the agent relative to in the absence of the agent. In a particular embodiment the PKA substrate is naturally occurring in the non-human mammal. In a preferred embodiments of this type the non-human mammal is a rodent. In a more preferred embodiment of this type the rodent is a mouse.
- 15 The *in vivo* method can further comprise administering the agent to a DARPP-32 knockout non-human mammal, *e.g.*, a homozygous DARPP-32 knockout mouse [*see* U.S. Patent No:5,777,195, Issued July 7, 1998; Fienberg *et al.*, *Science* **281**:838-842 (1998)] Preferably the agent is administered along with a dopamine D1 receptor agonist. The amount (and/or rate) of phosphorylation of the PKA substrate is then
- 20 determined. Since the administration of the dopamine D1 receptor agonist in the absence of the agent results in an increase in the phosphorylation state of a PKA substrate, an agent is identified as capable of modulating the phosphorylation state of Thr75 DARPP-32 when the amount (and/or rate) of phosphorylation of the substrate is not significantly changed in the presence of the agent relative to in the absence of
- 25 the agent. In a particular embodiment the PKA substrate is naturally occurring in the non-human mammal.

Alternatively, or in conjunction with the above *in vivo* and/or *in situ* and/or *in vitro* methods the locomotor activity of the animal subject, for example, can be determined as described in Example 2 below.

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Preferably the agent identified by a method of the present invention can cross and more preferably readily pass through the blood brain barrier. Alternatively, the agent can be modified or otherwise altered so that it can cross or be transported across the
5 blood brain barrier.

As would be clearly understood by a person of ordinary skill in the art, any and/or all of the above embodiments for identifying an agent (or drug) that can modulate the phosphorylation state of Thr75 DARPP-32 including such procedures that incorporate rational drug design, as exemplified below, can be combined to form additional drug
10 screens and assays, all of which are contemplated by the present invention.

In addition, the present invention provides methods for treating dopamine dysregulation in an individual (*e.g.*, a patient) or an animal subject. One such embodiment comprises administering to the individual an agent that inhibits the phosphorylation of Thr75-DARPP-32. In another embodiment the agent promotes the
15 dephosphorylation of Thr75-DARPP-32. In a particular embodiment, the method treats dopamine dysregulation that is related to a symptom and/or disease state characteristic of schizophrenia. In another embodiment the method treats dopamine dysregulation that is related to a symptom and/or disease state characteristic of Parkinson's Disease. In still another embodiment the method treats dopamine
20 dysregulation that is related to a symptom and/or disease state characteristic of Tourette's syndrome. In yet another embodiment the method treats dopamine dysregulation that is related to a symptom and/or disease state characteristic of Huntington's disease. In still another embodiment the method treats dopamine dysregulation that is related to a symptom and/or condition characteristic of drug
25 abuse. In a preferred embodiment the method treats dopamine dysregulation that is related to a symptom and/or condition characteristic of attention deficit hyperactivity. Preferably the agent can cross through the blood brain barrier in sufficient quantities and at a sufficient rate so as to allow the treatment of the dopamine dysregulation and thereby the condition or disease. In one such embodiment the agent is administered

In a particular embodiment, the phosphorylation of Thr75-DARPP-32 is inhibited by inhibiting Cdk5. In a preferred embodiment the agent is roscovitine. In another

Accordingly, it is a principal object of the present invention to provide methods of treating diseases and/or conditions that are effected by the DARPP-32/Protein

It is a further object of the present invention to provide methods of identifying agents/drugs that can modulate the phosphorylation state of Thr75 DARPP-32.

It is a further object of the present invention to provide kits containing such reagents.

It is a further object of the present invention to provide a method of administering an agent, preferably identified by the methods disclosed herein, that can ameliorate a symptom of a dopamine-related disease and/or condition.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a-1e show the phosphorylation of DARPP-32 at Thr75 by Cdk5 *in vitro* and *in vivo*. Figs. 1a -1b show the phosphorylation of DARPP-32 by striatal Cdk5. Cdk5 was immunoprecipitated, as described [Tsai *et al.*, *Development* **119**:1029-1040 (1993)], from 100 μ g of acutely dissected striatal homogenate prepared from wild type (lanes 1-4) or p35^{-/-} (lane 5) mice. The immunoprecipitated complex from wild type mice contained both Cdk5 and its cofactor p35, as determined by immunoblotting (antibodies from Santa Cruz Biotechnology,). Roscovitine (50 μ M), a selective Cdk5 inhibitor, was included as indicated. The precipitates were used to phosphorylate purified recombinant DARPP-32 at a dilution of 1:20. Reaction mixtures containing 600 μ Ci/ml [γ -³²P]ATP were incubated for 0 min (lane 1) or 60 min (lanes 2-5), followed by addition of gel loading buffer (final concentrations, 1% SDS, 12% glycerol, 25 mM DTT), and boiling for 5 min. Samples were subjected to SDS-PAGE (15% acrylamide), gels were dried, and radiographic images were generated using a PhosphorImager (Molecular Dynamics). Fig. 1c shows the phosphorylation of DARPP-32 at Thr75 by recombinant Cdk5. Reaction mixtures containing 10 μ M DARPP-32, 200 μ M ATP, 30 mM MOPS (pH 7.2), and 5 mM MgCl₂ were incubated for 60 min in the absence or presence of 30 μ g/ml each of partially purified GST-Cdk5 and GST-p25 (active fragment of p35) at 30°C. Reaction mixtures were processed as above, followed by electrophoretic transfer to nitrocellulose and immunoblotting using a phospho-Thr75 phosphorylation state-specific antibody prepared as described [Czernik *et al.*, in *Regulatory Protein Modification* (ed., H.C. Hemming, Jr) Pg. 219-246 (Humana, Tototwa NJ (1997))]. Fig. 1d-1e show the phosphorylation of DARPP-32 at Thr75 in intact striatum. Striatal tissue from wild type (lane 1) or p35^{-/-} (lane 2) mice was homogenized by sonication in boiling 1% SDS and 50 mM NaF. Equal amounts of protein were

processed as described above and immunoblots were probed with antibodies to phospho-Thr75 DARPP-32 and total DARPP-32.

Figures 2a-2g show the inhibition of PKA by phospho-Thr75 DARPP-32. Figs. 2a-2c show the effect of roscovitine on phospho-Thr75 and phospho-Thr34. Mouse striatal slices were prepared and incubated in the absence (Con.) or presence (Ros.) of 10 μ M roscovitine for 1 hour, using standard methodology [Snyder *et al.*, *J. Neurosci.* **12**:3071-3083(1992)]. Tissue was processed as described in the legend to Fig. 1a-1e and immunoblotted for phospho-Thr75 (Fig. 2a), total DARPP-32 (Fig. 2b), or phospho-Thr34 (Fig. 2c). Fig. 2d shows the phosphorylation by PKA of dephospho-DARPP-32 and phospho-Thr75 DARPP-32. Purified DARPP-32 was phosphorylated to a stoichiometry of 0.93 mol/mol using native cdk1/cyclin B purified from sea star. Phospho-Thr75 DARPP-32 was precipitated from the reaction mixture with 5% TCA, resuspended in 1 M Tris-HCl (pH 8.8), and dialyzed against 10 mM HEPES (pH 7.4). Dephospho- and phospho-Thr75 DARPP-32 were phosphorylated by PKA in the presence of [γ - 32 P]ATP as previously described [Girault *et al.*, *J. Biol. Chem.* **264**:21748-21759 (1989)] and subjected to SDS-PAGE. Fig. 2d shows the radiographic image of 32 P-labeled DARPP-32; Fig. 2e shows the Coomassie brilliant blue stain; and Fig. 2f shows the quantitation by PhosphorImager analysis of phosphorylation by PKA of dephospho-DARPP-32 and Phospho-Thr75 DARPP-32. Fig. 2g depicts a Lineweaver-Burk kinetic analysis of PKA phosphorylation of ARPP-21 in the presence of 0 (\circ), 5 (\square), 10 (\diamond), or 15 (Δ) μ M Phospho-Thr75 DARPP-32. Reactions were carried out as described [Hemmings *et al.*, *J. Biol. Chem.* **264**:7726-7733 (1989)]. The insert in Fig. 2g shows the secondary plot from which the K_i value was derived.

Figures 3a-3h show the effect of reducing phospho-Thr75 DARPP-32 on PKA activity in intact neurons. Figs. 3a -3b show the effect of roscovitine on phosphorylation of GluR1 and ARPP-16. Striatal slices from either wild type or DARPP-32^{-/-} mice were incubated for 1 h in the absence (Con.) or presence (Ros.) of

- 10 μ M roscovitine. Homogenates were processed using 10-20% acrylamide gradient gels as described in the legend to Figs. 2a-2g and immunoblots were carried out with antibodies to phospho-Ser845 GluR1 or phospho-Ser88 ARPP-16. Figs. 3c-3f show the dopamine signaling in wildtype and p35^{-/-} mice. Striatal slices from wildtype and
- 5 p35^{-/-} mice were incubated for 10 min in the absence (Con.) or presence (D1) of 1 μ M SKF 81297, a selective D1 receptor agonist. Homogenates were processed as above and immunoblotting was carried out with antibodies directed against the PKA phosphorylation sites of the proteins indicated at the left of each panel
- phospho-Ser845 GluR1, n = 4; (phospho-Thr34 DARPP-32, n = 6; phospho-Ser55
- 10 ARPP-21, n = 4; and phospho-Ser88 ARPP-16, n = 2; *p < 0.05 versus control, †p < 0.05 versus wt D1, by ANOVA with Newman-Kuel post-hoc test). Figs. 3g and 3h show the effect of roscovitine on whole cell Ca²⁺ current. Fig. 3g is a plot of peak Ca²⁺ current versus time in striatal neurons. Fig. 3h is a box-plot summary of the roscovitine-induced increase of Ca²⁺ current. Peak voltage-gated Ca²⁺ current in
- 15 acutely dissociated striatal neurons was recorded from wildtype and DARPP-32^{-/-} mice in response to bath application of 10 μ M roscovitine (shaded area). Standard whole-cell patch-clamp techniques were used as described [Surmeier *et al.*, *Neuron* 14:385-397 (1995)].
- 20 Figure 4A depicts a model illustrating dual effects of phosphorylation of DARPP-32 in regulation of PKA and PP1 activities. Dopamine D1 receptor activation of PKA and phosphorylation of Thr³⁴ by PKA converts DARPP-32 into an inhibitor of PP1. This activation of PKA and inhibition of PP1 contributes synergistically to increased phosphorylation of various substrates including GluR1 AMPA receptors, NR1 NMDA
- 25 receptors, and calcium channels. Conversely, phosphorylation of DARPP-32 at Thr75 by Cdk5 causes inhibition of PKA and activation of PP1. Figure 4B displays potential therapeutic targets to modulate the phosphorylation state of Threonine-75 (Thr75) of DARPP-32. Modulators of first messengers, neurotransmitters or signal transduction steps upstream of Cdk5 are at one end of the spectrum. Such modulators are
- 30 anticipated to effect Cdk5 regulatory factors, which in turn, can effect the Cdk5/Nck5a catalyzed phosphorylation of DARPP-32 at Thr75. Phosphorylation of

DARPP-32 at Thr75 by Cdk5 causes inhibition of PKA, while dephosphorylation is catalyzed by PP2A .

Figure 5 (PRIOR ART) displays the chemical structures of olomucine, flavopiridol, staurosporine and Paullone which inhibit Cdk5 kinase activity by competitively

5 binding to the ATP binding site.

Figures 6A-6F schematically show the steps involved in the generation of phosphorylation state-specific antibodies (PSSAb) to Thr75 DARPP-32. Figure 6A depicts a short synthetic phospho-peptide corresponding to the region surrounding the target site, phospho-Thr75 DARPP-32. Figure 6B schematically depicts the

10 conjugation of the phospho-peptide to a carrier protein (in this example, *Limulus* Hemocyanin) and the preparation for inoculation of the rabbit. Figure 6C represents the inoculation, boosting and bleeding of rabbits to obtain the sera containing the desired antibody. Typical results of phospho-peptide immuno-dot-blot screening of serum are shown in Figure 6D. Figure 6E represents the purification of the phospho-specific antibody (Ab). The purification used Protein A and

15 phospho/dephosphopeptide affinity chromatography [Czernik *et al.*, in *Regulatory Protein Modification* (ed., H.C. Hemming, Jr) Pg. 219-246 (Humana, Tototwa NJ (1997))]. Figure 6F shows immunoblot results confirming the selectivity of the antibody for phosphorylated protein.

20 Figure 7 shows the alignment of the amino acid sequences of various DARPP-32 isoforms (bovine, SEQ ID NO:4; rat, SEQ ID NO:3; and mouse, SEQ ID NO:2). The amino acid sequences as shown are deduced from their corresponding cDNA nucleic acid sequences. The alignments include spaces introduced by dashes where necessary. The numbers refer to the position of amino acids relative to the bovine

25 sequence. The total number of amino acids is indicated at the end of each sequence. Nonconserved amino acid differences are shown in bold. Underlined sequences represent synthetic peptides that have been used to generate antibodies, including those that are phosphorylation state-specific. The phospho-peptide used to generate

Figures 8A-8C show the increased expression of Cdk5 in inducible transgenic mice overexpressing Δ FosB and in rats chronically treated with cocaine. Fig. 8A shows the cDNA expression arrays probed with radiolabeled cDNA from control mice ($-\Delta$ FosB) (left) or transgenic mice overexpressing Δ FosB ($+\Delta$ FosB) (right). The positions of oligonucleotides encoding Δ FosB and Cdk5 are indicated. Fig. 8B shows the comparison of the levels of Cdk5 gene expression by *in situ* hybridization. Representative labelings are shown for sections from inducible transgenic mice on (Dox) or off doxycycline (H_2O) (left) and rats treated chronically with saline or cocaine (right). Quantitation of signals in the caudatoputamen (CP) and nucleus accumbens (NA) are shown. Fig. 8C shows the comparison of the levels of Cdk5 protein. Representative Cdk5 immunoblots of striatal tissue dissected from inducible transgenic mice on or off doxycycline (left) and from rats treated chronically with saline or cocaine (right) are shown with quantitation. Data represent means \pm SEM for $n = 6$; $*p < 0.05$ compared to control, Student's t test.

Figures 9A-9C show the effect of Cdk5 inhibitors on locomotor behavioral response to repeated cocaine injections. Fig. 9A shows the locomotor activity for rats, given intra-accumbens infusions of saline or roscovitine and i.p. injections of saline or cocaine, from 50 to 60 min post-injection on successive days 1-5. Fig. 9B shows the behavioral effects of each treatment on day 5 measured for 60 minutes post injection with values plotted at 10 min intervals. Fig. 9C shows the behavioral effects of intra-accumbens infusion of olomoucine (left panel) or the inactive analog, iso-olomoucine on cocaine (i.p.)-induced locomotor activity from 40-50 min post-injection at days 1 and 3 of treatment. All data represent mean activity counts \pm SEM measured by photocell over test period, * $p < 0.05$, ** $p < 0.001$ by ANOVA and post hoc Sheffe's F-test. For day 3 in panel A, $p < 0.07$. For panels A and B, Sal/Sal, $n = 6$; Sal/Coc, $n = 11$; Sal/Coc, $n = 9$; Ros/Coc, $n = 11$. For panel C, Sal/Sal, $n = 6$, Sal/Coc, $n = 9$, Olo/Coc, $n = 11$; Iso/Coc, $n = 11$.

Figures 10A-10C show increased phosphorylation of DARPP-32 by Cdk5 in inducible transgenic mice overexpressing Δ FosB and in rats chronically treated with cocaine. Fig. 10A shows the quantitative immunoblot analysis of level of phospho-Thr75 DARPP-32 in striatal tissue dissected from inducible Δ FosB transgenic mice on or off doxycycline. Representative blots are shown for phospho-Thr75 DARPP-32 and total DARPP-32 in the top two panels and quantitation is shown in the bottom panel. Figs. 10B and 10C show the level of phospho-Thr75 DARPP-32 in caudatoputamen (Fig. 10B) and nucleus accumbens (Fig. 10C) from rats treated with saline or chronic cocaine administration for 10 days. Data represent means \pm SEM for $n = 6$; $*p < 0.05$, Mann Whitney nonparametric t test.

Figures 11A-11C show the effects of chronic cocaine exposure on dopamine/PKA signalling in the striatum. Fig. 11A shows the effect of the selective D_1 agonist SKF 81297 (D_1), in comparison to untreated controls (Con), on PKA phosphorylation of DARPP-32 (left), ARPP-21 (middle), and ARPP-16 (right) in striatal slices from saline and cocaine-treated rats. Fig. 11B shows the effects of chronic cocaine on PKA phosphorylation of DARPP-32 and GluR1 in striatal homogenates. Data in Figs. 11A and 11B represent means \pm SEM, $*p < 0.01$ versus controls, Mann Whitney nonparametric t test, $n = 6$. Fig. 11C shows the effect of chronic cocaine on ligand-gated AMPA current. Kainate-sensitive AMPA current recordings in saline-treated or cocaine-treated rats (left) and statistical analyses (right) are shown. Data represent means \pm SEM, $*p < 0.01$ versus controls, unpaired t test, $n = 6$.

DETAILED DESCRIPTION OF THE INVENTION

As disclosed herein, a single protein can, dependent on the particular amino acid residue phosphorylated, function either as a kinase or phosphatase inhibitor. DARPP-32 (Dopamine and cyclic AMP-Regulated Phospho-Protein, M_r 32 kDa) is known to be converted into an inhibitor of protein phosphatase 1 (PP1) when phosphorylated by cAMP dependent protein kinase (PKA) at threonine-34 (Thr34). Indeed, schizophrenia can be treated by inhibiting the dephosphorylation of

thr³⁴- phosphorylated DARPP-32 [U.S. Patent 6,013,621, Issued January 11, 2000, the contents of which are hereby incorporated by reference in their entireties.] The present invention shows that DARPP-32 can also be phosphorylated at threonine-75 (Thr75) by cyclin-dependent kinase 5 (Cdk5) and that Cdk5 catalyzed

- 5 phosphorylation of Thr-75 DARPP-32: (i) prevents DARPP-32 from acting as a substrate for PKA; (ii) converts DARPP-32 into an inhibitor of the PKA, including inhibiting the ability of PKA to phosphorylate other PKA substrates; and (iii) prevents DARPP-32 from being converted to an inhibitor of PP1.

- 10 Cdk5 phosphorylates DARPP-32 *in vitro* and in intact brain cells. Phospho-Thr75 DARPP-32 protein inhibits PKA *in vitro* by a competitive mechanism. Reduction of phospho-Thr75 DARPP-32 in striatal slices, either by a Cdk5 specific inhibitor or by the use of genetically altered mice, results in increased phosphorylation of PKA substrates and augmented peak voltage-gated Ca²⁺ currents. Thus DARPP-32 is a
- 15 bifunctional signal transduction molecule which, by distinct mechanisms, controls a major serine/threonine kinase as well as a major serine/threonine phosphatase.

- In its capacity as a modulator of DARPP-32 phosphorylation, Cdk5 may play a role in Huntington's disease, Parkinson's disease, schizophrenia, Tourette's syndrome, drug abuse, and attention deficit disorder. Indeed, as disclosed herein, Cdk5 plays a key
- 20 role in DARPP-32 activity, thereby, identifying a whole new set of targets (*see* Fig.4a), which may serve as the basis for the identification/development of therapeutic agents for dopamine related disorders. Indeed, roscovitine, a specific Cdk5 inhibitor, increases locomotor behavior in rats which is the predicted effect of inhibition of Cdk5 phosphorylation of Thr75 of DARPP-32, thus demonstrating the potential of
 - 25 Cdk5 inhibitors to modulate dopamine dysregulation.

Furthermore, Example 2 below discloses a homeostatic response mechanism to chronic cocaine exposure in which Δ FosB-mediated changes in gene expression are coupled to the regulation of Cdk5 and dopamine signaling in striatal neurons. Thus, Cdk5 is shown to be a downstream target gene of the transcription factor Δ FosB,

which accumulates in dopaminoceptive neurons of the striatum with chronic exposure to cocaine. Therefore, as described in Example 2 below, Δ FosB overexpression, and/or chronic cocaine administration raises Cdk5 mRNA, Cdk5 protein levels, and Cdk5 activity in the striatum in mice. Moreover, intra-striatal injection of Cdk5 inhibitors potentiated behavioral effects of repeated cocaine administration. These results demonstrate that Δ FosB-mediated changes in Cdk5 levels and resulting alterations in D₁ dopaminergic signaling contribute to adaptive changes in the brain related to cocaine addiction.

Therefore, the present invention provides DARPP-32 that has been phosphorylated at threonine-75 as well as fragments of DARPP-32 comprising phospho-Thr75 DARPP-32. The present invention further provides methods of treating dopamine dysfunction in animals, preferably in humans, by administering to the animal an agent that either inhibits the phosphorylation of Thr75-DARPP-32 or promotes the dephosphorylation of Thr75-DARPP-32. Preferably, the agent can cross, and more preferably readily pass through, the blood brain barrier.

The present invention further provides methods of identifying therapeutic agents that are designed to be more specific and thereby less likely to produce deleterious side-effects. Indeed, any compound that is identified as modulating the level of phosphorylation of threonine-75 of DARPP-32 or modulating the functional activity of DARPP-32 that is phosphorylated on threonine-75 is a potential drug for treating dopamine-related disorders. Thus, the present invention provides methods of identifying potential regulators of Cdk5 kinase activity.

The present invention further provides new methodology for identifying compounds that modulate phospho-Thr75 DARPP-32 concentrations that can be used in the treatment of dopamine-related diseases. Therefore, the present invention also provides DARPP-32 that has been phosphorylated at threonine-75, which may be used as a standard in such drug assays or as an inhibitor of PKA either *in vitro* and/or *in vivo*. Similarly, peptide fragments of phosphoThr75 DARPP-32 which contain the

phosphorylated Thr75 may also serve as standards in such drug assays or as competitive inhibitors of the Cdk5 catalyzed phosphorylation of DARPP-32 are also provided.

Therefore, if appearing herein, the following terms shall have the definitions set out
5 below.

As used herein the term "DARPP-32" is used interchangeably with "Dopamine- and cyclic AMP (cAMP)-Regulated PhosphoProtein" and is a 32 kilodalton cytosolic protein that is selectively enriched in medium-sized spiny neurons in neostriatum. The human DARPP-32 has the amino acid sequence is SEQ ID NO:1; the mouse
10 DARPP-32 has the amino acid sequence is SEQ ID NO:2; the rat DARPP-32 has the amino acid sequence is SEQ ID NO:3; and the bovine DARPP-32 has the amino acid sequence is SEQ ID NO:4. The alignment of the latter three sequences are shown in Figure 7.

As used herein the term "Thr75 DARPP-32" is used interchangeably with
15 "thr⁷⁵ DARPP-32", "Threonine-75 DARPP-32" and "threonine-75 DARPP-32" along with analogous abbreviations, and denotes the seventy-fifth amino acid residue in the amino sequence of DARPP-32 as disclosed by Brene *et al.* [*J. Neurosci.* **14**:985-998 (1994)] having the GenBank Accession of AAB30129.1 (SEQ ID NO:1) which is a threonine residue that, as disclosed herein, can be phosphorylated by Cdk5.

20 As used herein the term "phospho-Thr75 DARPP-32" or denotes the phosphorylated form of Thr75 DARPP-32.

As used herein the term "Thr34 DARPP-32" is used interchangeably with
"thr³⁴ DARPP-32" "Threonine-34 DARPP-32" and "threonine-34 DARPP-32" along with analogous abbreviations and denotes the thirty-fourth amino acid residue of the
25 amino sequence of DARPP-32 as disclosed by Brene *et al.* [*J. Neurosci.* **14**:985-998 (1994)] having the GenBank Accession of AAB30129.1 (SEQ ID NO:1) which is a

threonine residue that can be phosphorylated by the cyclic AMP dependent protein kinase (PKA).

As used herein “CDK5”, “Cdk5” or “cdk5” are used interchangeably with “cyclin-dependent kinase 5” which is also known as neuronal cyclin-dependent-like protein (Nclk) and tau protein kinase II (TPKII). Cdk5 is a member of the cyclin-
 5 dependent kinases but atypically Cdk5 employs a non-cyclin cofactor called neuronal cyclin-dependent-like kinase 5 associated protein (Nck5a) rather than a cyclin. When the term “Cdk5” is used in descriptions of kinase reactions it should be understood that the active form, *i.e.*, the “Cdk5/Nck5a complex” (see below) may be the actual
 10 catalytic factor and/or a fragment of Cdk5 which retains at least 10% of the catalytic activity of Cdk5.

As used herein “an analog of Cdk5” is used interchangeably “a homolog of Cdk5” and is a protein kinase that like Cdk5 phosphorylates DARPP-32 on Threonine-75 but not on Threonine-34. One such analog is cdk1.

15 As used herein “Nck5a” is used interchangeably with “neuronal cyclin-dependent-like kinase 5 associated protein” and is a non-cyclin cofactor for Cdk5. There are at least two isoforms of Nck5a in the brain (p35 and p39) which may also exist as proteolytic fragments (*i.e.*, p25 and p29, respectively).

As used herein the term “Cdk5/Nck5a complex” denotes the complex formed between
 20 Cdk5 and Nck5a which is an active form of the Cdk5 kinase.

As used herein the term “Cdk5 phosphorylatable fragment of DARPP-32” is a protein fragment of DARPP-32 that contains a threonine residue that when in the dephosphorylated form can be phosphorylated by Cdk5. For human DARPP-32 having the amino acid sequence of SEQ ID NO:1, the threonine residue is preferably
 25 Thr75 DARPP-32. Such fragments can be between about 5 to 100 residues, or more preferably between about 10 and 50 residues. For example, in a particular

embodiment the peptide fragment comprises 5 consecutive amino acids from SEQ ID NO:1 including Thr75. In another embodiment of this type, the peptide fragment comprises 7 consecutive amino acids from SEQ ID NO:1 including Thr75. In an alternative embodiment the peptide fragment comprises between 10 to 25 consecutive amino acids from SEQ ID NO:1 including Thr75. All of the peptide fragments can be part of fusion peptides or proteins. A Cdk5 phosphorylatable fragment of DARPP-32 can be prepared by phosphorylating the dephosphorylated fragment or by cleaving (such as with a protease) the phosphorylated fragment from a larger fragment of phospho-Thr75 DARPP-32 protein or from the full-length phospho-Thr75 DARPP-32 protein. Thus the fragments can be synthesized by either standard peptide synthesis described below, or generated through recombinant DNA technology or by classical proteolysis.

As used herein the amount and/or rate of phosphorylation of DARPP-32 or the Cdk5 phosphorylatable fragment of DARPP-32 in a kinase reaction is “significantly changed” when the amount and/or rate of phosphorylation of DARPP-32 or the Cdk5 phosphorylatable fragment of DARPP-32 is increased or decreased by at least about 10- 25%, relative to the control reaction. Preferably, a significant change in rate of the phosphorylation of DARPP-32 by Cdk5 for example, observed in the presence of a potential modulator is at some point correlated with the Michaelis constants (*e.g.*, the V_{max} or K_m) of the reaction. For example, in the case of an inhibitor a K_i can be determined. Thus it may be necessary to study various concentrations of a modulator in a reaction mixture to allow the identification of the potential modulator as a modulator.

As used herein the term “dopamine dysregulation” is used interchangeably with the term “dopamine-related disorder” and specifically includes “dopamine-related diseases”. A dopamine-related disorder can be a disease (*e.g.*, Parkinson’s disease) or a condition (*e.g.*, addiction to cocaine) that involves an aberration or dysregulation of a pathway effected by dopaminergic neurotransmission in the brain. Preferably the pathway effected includes the phosphorylation and/or dephosphorylation of DARPP-

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As used herein the term “about” means within 10 to 15%, preferably within 5 to 10%. For example an amino acid sequence that contains about 60 amino acid residues can

contain between 51 to 69 amino acid residues, more preferably 57 to 63 amino acid residues.

Drug Screening

- The present invention provides a number of viable targets for screening drugs that can
- 5 modulate Thr75 DARPP-32 phosphorylation and thereby ameliorate dopamine-related disorders. Potential modulators include small organic molecules that mimic the function of first messengers, and/or analogs thereof, inhibitors, and/or toxins that modulate the processes that effect the phosphorylation of Thr75 DARPP-32 such as represented schematically in Figure 4B.
- 10 One particular target for regulating Thr75 DARPP-32 phosphorylation is Cdk5. Thus, in one drug screen of the present invention compounds are identified that effect the phosphorylation of DARPP-32 by Cdk5. In one such embodiment, Cdk5 is added to the reactions that include a drug candidate, ATP, buffering reagents, and an appropriate substrate (*e.g.*, DARPP-32 or a fragment of DARPP-32 encompassing
- 15 Thr75). In a particular embodiment, one or more kinases that do not phosphorylate Thr75 DARPP-32, such as MAP kinase (*see below*) are also employed (preferably purified and demonstrated to have *in vitro* activity) as controls. These kinases can then be added to analogous reactions that include the drug candidate, ATP, buffering reagents, and an appropriate kinase substrate. Combinatorial libraries of chemical
- 20 compounds, based on different structural skeletons (*e.g.*, purines) as well as unrelated naturally occurring compounds can then be tested as drug candidates. In a preferred embodiment of this type, the assay is performed using high throughput technology with automated robotic technology. Positive results (or a "hit") represent either the reduced or increased phosphorylation of the kinase substrate by Cdk5, as compared to
- 25 the control reactions (in which the drug candidate is not included in the assay). Preferably the drug candidate has minimal to no effect on the phosphorylation level of the control kinase reactions examined.

Once a drug candidate is selected, structural variants of the drug candidate can be tested. These compounds can also be scrutinized and modified with parameters such as membrane permeability, specificity of effects, and toxicity. The selected inhibitors (e.g., the most potent) of this secondary screening can then be evaluated *in situ* and in animal models to determine if they alter phospho-Thr75 DARPP-32 concentrations and/or induce the predicted behavior alterations with minimal to no side-effects. Such behavioral abnormalities include testing locomotor activity, e.g., administration of drugs of abuse to mice result in increased locomotor activity [see, Kosten *et al.*, *J. Pharmacol., Exp. Ther.* **269**:137-144 (1994) and Example 2 below; and/or self-administration of selected drugs or in prepulse inhibition *see generally* U.S. Patent No:5,777,195 Issued July 7, 1998 hereby incorporated by reference in its entirety]. These tests can be then be followed by human trials in clinical studies. Alternatively, in certain instances, human trials in clinical studies can be performed without animal testing. Compounds affecting targets other than Cdk5 can also be similarly screened, using alternative targets exemplified below.

Alternatively, modulators of Thr75 DARPP-32 phosphorylation including inhibitors of Cdk5 (for example) can be obtained by screening a random peptide library produced by recombinant bacteriophage for example, [Scott and Smith, *Science*, **249**:386-390 (1990); Cwirla *et al.*, *Proc. Natl. Acad. Sci.*, **87**:6378-6382 (1990); Devlin *et al.*, *Science*, **249**:404-406 (1990)] or a chemical library. Using the "phage method" very large libraries can be constructed (10^6 - 10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method [Geysen *et al.*, *Molecular Immunology* **23**:709-715 (1986); Geysen *et al.* *J. Immunologic Method* **102**:259-274 (1987)] and the method of Fodor *et al.* [*Science* **251**:767-773 (1991)] are examples. Furka *et al.* [*14th International Congress of Biochemistry, Volume 5*, Abstract FR:013 (1988); Furka, *Int. J. Peptide Protein Res.* **37**:487-493 (1991)], Houghton [U.S. Patent No. 4,631,211, issued December 1986] and Rutter *et al.* [U.S. Patent No. 5,010,175, issued April 23, 1991] describe methods to produce a mixture of peptides that can be tested as modulators of Thr75 DARPP-32 phosphorylation.

- In another aspect, synthetic libraries [Needels *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:10700-4 (1993); Ohlmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* **90**:10922-10926 (1993); Lam *et al.*, International Patent Publication No. WO 92/00252; Kocis *et al.*, International Patent Publication No. WO 9428028, each of which is incorporated
- 5 herein by reference in its entirety], and the like can be used to screen for modulators of Thr75 DARPP-32 phosphorylation such as an inhibitor of Cdk5, for example, according to the present invention. Once a potential modulator is identified, chemical analogues can be either selected from a library of chemicals as are commercially available from most large chemical companies including Merck, GlaxoWellcome,
- 10 Bristol Meyers Squib, Monsanto/Searle, Eli Lilly, Novartis and Pharmacia UpJohn, or alternatively synthesized *de novo*. The prospective agent (drug) can be placed into any standard assay to test its effect on the phosphorylation of Thr75 of DARPP-32 by Cdk5 for example. A drug is then selected that modulates the phosphorylation state of Thr75 of DARPP-32.
- 15 The present invention also contemplates screens for small molecules, analogs thereof, as well as screens for natural modulators of Thr75 DARPP-32 phosphorylation such as those that bind to and inhibit Cdk5 *in vivo*. Thus, an analog of roscovitine, which is known to competitively inhibit the binding of ATP to the ATP binding domain in the catalytic site of Cdk5, can be tested in order to obtain a modulator having a more
- 20 specific and/or potent effect than roscovitine,. Alternatively, natural products libraries can be screened using assays of the invention for molecules that antagonize Cdk5 activity. Other families of drugs that have been shown to inhibit Cdk5 include paullones [Leost *et al.*, *Eur. J. Biochem.* **267**:5983-5994 (2000) the contents of which are hereby incorporated by reference in their entirety] and indirubins [Leclerc *et al.*,
- 25 *J. Biol. Chem.* (epub ahead of print Sept. 29, 2000) the contents of which are hereby incorporated by reference in their entirety]

In one particular assay the target *e.g.*, Cdk5 can be attached to a solid support. Methods for placing Cdk5 on the solid support are well known in the art and include such things as linking biotin to Cdk5 and linking avidin to the solid support. The

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embodiment, the amount and/or rate of the phosphorylation is determined of Thr75 DARPP-32 or a fragment of DARPP-32 comprising the threonine-75 residue. For such assays labeled [γ - ^{32}P]ATP can be used. In a particular embodiment a phosphorylation state specific antibody for Thr75 DARPP-32 can be employed. The determination can

5 include a real-time determination, or alternatively, aliquots from the incubation mixture can be withdrawn at defined intervals and the aliquots can be subsequently placed on nitrocellulose paper or on gels. In a particular embodiment the potential modulator is selected when it is an inhibitor of the phosphorylation reaction.

In an assay in which the Thr75 DARPP-32 phosphorylation is being measured

10 indirectly, *e.g.*, for an effect of the phospho-Thr75 DARPP-32 protein, a control in which the recombinant protein containing a Thr75Ala mutation of DARPP-32 can be used to confirm that the phosphorylation of Thr75 DARPP-32 is involved in the effect.

Thus, a modulator that inhibits, or in the alternative stimulates the phosphorylation is then selected. In a particular embodiment, the effect of a potential modulator on the

15 catalytic activity is determined. The potential modulator can then be tested for its effect on the physiological consequence of PKA inhibition by phospho-Thr75 DARPP-32. For this purpose, voltage-gated Ca^{2+} currents, (which are known to be regulated by PKA) can be analyzed using patch-clamp recordings of dissociated striatal neurons (*see e.g.*, Figs. 3g and 3h) in the presence and absence of the potential

20 modulator of Thr75 DARPP-32 phosphorylation. The whole-cell Ca^{2+} current in the neuron can then be determined. When an increase in the Ca^{2+} current is determined in the presence of the potential modulator relative to in its absence, the potential modulator is selected/identified as an inhibitor of Thr75 DARPP-32 phosphorylation, whereas when a decrease in the Ca^{2+} current is determined in the presence of the

25 potential modulator relative to in its absence, the potential modulator is selected/identified as an agonist of Thr75 DARPP-32 phosphorylation. Such assays can further include testing the potential modulator with striatal neurons from DARPP-32 knockout mice, in which a potential modulator is selected when it shows no effect of the PKA dependent voltage-gated Ca^{2+} currents. Similarly, P35^{-/-} mouse striatal

neurons can be employed to ascertain the role of Cdk5 in the effect (as described in Example 1, below).

- Alternatively, or in conjunction with the above assays, a potential modulator can be added to striatal tissue slice (as exemplified below). Tissue samples can be treated
- 5 with various concentrations of a potential modulator and the sample can then be analyzed for DARPP-32 phosphorylation of either threonine-75 or threonine-34. Potential modulators that have the potential to affect phospho-Thr75 (*e.g.*, by affecting one of the processes indicated in Fig. 4B), can be tested for example, on intact neurons *in situ* by treatment of acutely prepared neostriatal slices incubated in Kreb's
- 10 bicarbonate buffer solution containing the reagent. The effects of these compounds can be tested by empirically defining the optimal concentration and time of incubation.

- Similarly, alternatively, or in conjunction with the above assays an animal model can be used to ascertain the effect of a potential agent on a dopamine related condition. A potential modulator that ameliorates the dopamine related condition can then be
- 15 selected [*see* U.S. Patent No:5,777,195, Issued July 7, 1998; Fienberg *et al.*, *Science* **281**:838-842 (1998), the contents of each are hereby incorporated by reference herein, in their entireties]. For example, locomotor behavioral response of the animal can be determined in the presence and absence of the agent. Since, the phosphorylation of Thr75 DARPP-32 (i) prevents DARPP-32 from acting as a substrate for PKA; (ii)
- 20 converts DARPP-32 into an inhibitor of the PKA, including inhibiting the ability of PKA to phosphorylate other PKA substrates; and (iii) prevents DARPP-32 from being converted to an inhibitor of PP1, the phosphorylation of Thr75 DARPP-32 can be assumed to potentiate an effect that is opposed to that of the well characterized phospho-Thr34 DARPP-32.

- 25 Methods of testing a potential therapeutic agent (*e.g.*, a candidate drug, potential modulator, etc.) in an animal model are well known in the art. Thus potential therapeutic agents can be used to treat whole animals. The potential modulators can be administered by a variety of ways including topically, orally, subcutaneously, or

intraperitoneally (such as by intraperitoneal injection) depending on the proposed use. Optimal dose will be empirically defined. Animals can be sacrificed by focused microwave beam irradiation, for example. Striatal tissue can then be dissected and homogenates can be subjected to immunoblot analysis. An alternative approach that

5 can be employed assesses the potential efficacy of these compounds in relieving dopamine related pathological symptoms in animal models for disease. For example, treatment of rats or mice with 6-hydroxydopamine results in loss of dopaminergic afferent neurons, administration of quinolinic acid causes lesion of intrinsic striatal neurons, and MPTP destroys dopamine containing nerve terminals. All of these cause

10 movement disorders and serve as animal models for disorders in dopaminergic neurotransmission. Transgenic animals ectopically expressing the human disease causing form of the Huntington's disease (HD) gene exhibit neuropathological symptoms similar to those of HD patients. Models such as these can be used to assess the efficacy of any potential therapeutic agents. Generally, at least two groups of

15 animals are used in the assay, with at least one group being a control group which is administered the administration vehicle without the potential modulator.

There are also a number of potential regulators of Cdk5 activity that can be used as templates for designing potential drug analogs, or alternatively as potential targets for drug assays. Both the ribosomal complex protein, L34, and the DNA binding protein,

20 dbPA have previously been shown to bind and inhibit Cdk5. The neuronal secretory vesicle protein nsec (p67, Munc 18) has also been reported to activate Cdk5. Cdk T14 kinase as well as other kinases including casein kinase I have been implicated in the phosphorylation and regulation of Cdk5 [Sharma *et al.*, *Proc. Natl. Acad. Sci., USA* **96**:11156-11160 (1999)]. In addition, p35 has been implicated as a modulator of

25 Cdk5. Interestingly, p35 may be inhibitory whereas, a proteolytically processed fragment of p35 may be activating. In any case, the concentration of p35 in a cell may be the primary mode for regulating Cdk5 activity. Since p35 levels may be regulated through phosphatase-dependent activation of proteosomes, stimulating or inhibiting phosphatase-dependent activation of proteosomes in the striatum could potentially alter

30 the phospho-Thr75 DARPP-32 levels and thereby ameliorate dopamine related

disorders. Therefore, such factors can be envisioned as potential targets for the drug assays and screens presented herein.

As disclosed herein, when DARPP-32 is phosphorylated at Thr75 it cannot be phosphorylated by PKA. This phosphorylation of DARPP-32 at Thr-75 could alter its biochemical characteristics so that it can bind PKA, possibly near the active site, in a manner and with adequate affinity such that the PKA substrate site, Thr34 of DARPP-32, cannot come into proximity with the catalytic site of PKA. As a result of this phosphorylation-dependent interaction, not only is PKA unable to phosphorylate DARPP-32 but the activity of PKA toward other substrates may be inhibited in a competitive manner. Deletion mutants of DARPP-32 can be generated to define the inhibitor domain. Small phosphopeptides encompassing the Thr75 site may bind to PKA competitively (displacing holophospho-Thr75 DARPP-32) but may have no effect on PKA activity. These peptides could thus serve as the basis for the design of compounds which have the ability to interrupt the PKA inhibitory actions of phospho-Thr75 DARPP-32 *in vivo*.

In addition, as stated above, DARPP-32 is converted into an inhibitor of PP1 by phosphorylation at Thr34. DARPP-32 is also phosphorylated at Ser102 by casein kinase II (CKII) and at Ser 137 by casein kinase I (CKI). Phosphorylation at Ser102 improves the efficiency of phosphorylation of Thr34 by PKA. Phosphorylation of DARPP-32 at Ser137 reduces the efficiency of dephosphorylation of phospho-Thr34 by protein phosphatase 2B. Thus the net effects of phosphorylation of DARPP-32 at Ser102 and Ser137 by CKI and CKII, respectively, is to enhance the amount of time that DARPP-32 remains in its phospho-Thr34, or PP1 inhibitory state. The effect of phosphorylation of DARPP-32 at Thr75 upon phosphorylation kinetics at these other sites was therefore assessed. While phosphorylation of DARPP-32 by CKI and CKII was unaffected, the effect on PKA phosphorylation was quite dramatic. Thus when DARPP-32 is phosphorylated at Thr75 it may affect the dephosphorylation at any or all of the other 3 phosphorylation sites. DARPP-32 may also serve additional unknown functions and/or serve as a substrate for additional kinases. Phospho-T75 could exert

Activation of D1 dopamine receptors also appears to reduce phospho-T75 DARPP-32 levels. Phospho-Thr75 DARPP-32 is also dephosphorylated by protein phosphatase 2A (PP2A). PP2A dephosphorylation of Phospho-Thr75 DARPP-32 is itself regulated by PKA phosphorylation of a regulatory subunit of the PP2A phosphatase complex. (Known inhibitors PP2A include okadaic acid and calyculin A). PKA treatment of purified holo-PP2A appears to enhance PP2A dephosphorylation of phospho-T75 DARPP-32. Therefore, PP2A and more specifically the regulatory subunit of the PP2A are particular targets of the present invention for regulating the phosphorylation of Thr75 DARPP-32. In addition, other phosphatases such as PP4, may also dephosphorylate phospho-Thr75 DARPP-32 and therefore may serve as potential drug screen targets. Furthermore, phospho-Thr75 DARPP-32, as disclosed herein, is an inhibitor of PKA. Thus any compound identified that affects these interactions may also serve as the basis of a therapeutic treatment for dopamine-related diseases and therefore, all of the proteins that participate in such interactions may also be used in assays as described herein.

Furthermore, since the phosphorylation of Thr34 DARPP-32 and Thr75 DARPP-32 appear to be mutually exclusive, potential modulators of Thr75 DARPP-32 phosphorylation can be indirectly identified employing drug assays that measure for example, an *in vivo* effect of phospho-Thr34 DARPP-32, such as PP1 inhibition, PKA activity, the phosphorylation state of exogenous PKA substrates (see Example 1 below and Figures 2 and 3) or exogenous PP1 substrates. A potential inhibitor of Thr75 DARPP-32 phosphorylation is anticipated to enhance an effect stimulated by the phosphorylation of Thr34 DARPP-32, whereas a potential agonist of Thr75 DARPP-32

phosphorylation is anticipated to inhibit an effect stimulated by the phosphorylation of Thr34 DARPP-32.

Whereas at present no first messengers, neurotransmitters, or signal transduction steps that modulate Cdk5 activity have been reported (*see* Figure 4a), once these factors are identified they also can be used in the drug assays and screens that are exemplified herein, or alternatively in analogous assay procedures.

Protein-structure Based Design of Inhibitors of Cdk5:

The atomic coordinates for the cdk2-cyclinA-ATP complex have been determined [Jeffrey *et al.*, *Nature* **376**:313-320 (1995)] which has allowed the computer generated structural model of Cdk5 complexed with Nck5a (p25) and ATP to be compiled [Chou *et al.*, *Biochem. Biophys. Res. Comm.* **259**:420-428 (1999)]. Therefore, the present invention further provides a means of performing rational drug design to develop drugs that can modulate Thr75 DARPP-32 phosphorylation and thereby ameliorate dopamine related disorders. Such rational drug design can be performed using compounds that have been identified to inhibit Cdk5 as a starting point. Roscovitine, for example, has been exemplified below. Other compounds include olomucine, flavopiridol, staurosporine and paullone which are competitive inhibitors for ATP (Figure 5). Thus, the present invention provides screens and assays to allow more specific inhibitors to be identified.

Indeed, a potential modulator of Cdk5 can be examined through the use of computer modeling using a docking program such as GRAM, DOCK, or AUTODOCK [Dunbrack *et al.*, *Folding & Design*, **2**:27-42 (1997)], to identify potential modulators of Cdk5. These modulators can then be tested for their effect on Thr75 DARPP-32 phosphorylation. This procedure can include computer fitting of potential modulators to the Cdk5-Nck5a complex to ascertain how well the shape and the chemical structure of the potential modulator will bind to either Cdk5, Nck5a or to the Cdk5-Nck5a complex [Bugg *et al.*, *Scientific American*, **Dec.**:92-98 (1993); West *et al.*, *TIPS*, **16**:67-74 (1995)]. Computer programs can also be employed to estimate the attraction,

repulsion, and steric hindrance of the subunits with a modulator/inhibitor (*e.g.*, Cdk5-Nck5a complex and a potential destabilizer).

Generally the tighter the fit, the lower the steric hindrances, and the greater the attractive forces, the more potent the potential modulator since these properties are
 5 consistent with a tighter binding constant. Furthermore, the more specificity in the design of a potential drug the more likely that the drug will not interact as well with other proteins. This will minimize potential side-effects due to unwanted interactions with other proteins.

Initially compounds known to bind Cdk5, for example roscovitine, can be
 10 systematically modified by computer modeling programs until one or more promising potential analogs are identified. In addition systematic modification of selected analogs can then be systematically modified by computer modeling programs until one or more potential analogs are identified. Such analysis has been shown to be effective in the development of HIV protease inhibitors [Lam *et al.*, *Science* **263**:380-384
 15 (1994); Wlodawer *et al.*, *Ann. Rev. Biochem.* **62**:543-585 (1993); Appelt, *Perspectives in Drug Discovery and Design* **1**:23-48 (1993); Erickson, *Perspectives in Drug Discovery and Design* **1**:109-128 (1993)]. Alternatively a potential modulator could be obtained by initially screening a random peptide library produced by recombinant bacteriophage for example, *see* above. A peptide selected in this manner could then be
 20 systematically modified by computer modeling programs as described above, and then treated analogously to a structural analog as described above.

Once a potential modulator/inhibitor is identified it can be either selected from a library of chemicals as are commercially available from most large chemical companies including Merck, GlaxoWellcome, Bristol Meyers Squib, Monsanto/Searle,
 25 Eli Lilly, Novartis and Pharmacia UpJohn, or alternatively the potential modulator may be synthesized *de novo*. The *de novo* synthesis of one or even a relatively small group of specific compounds is reasonable in the art of drug design. The potential modulator

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For all of the drug screening assays described herein further refinements to the structure of the drug will generally be necessary and can be made by the successive iterations of any and/or all of the steps provided by the particular drug screening assay.

The present invention also provides kits for performing the methods of the present invention. One particular kit can be used in detecting the presence of phospho-Thr75 DARP-32 protein in a cellular sample. In one such embodiment the kit comprises a predetermined amount of a detectably labeled binding partner of the phospho-Thr75 DARP-32 protein. In a particular embodiment of this type the binding partner is a phosphorylation state-specific antibody to the phospho-Thr75 DARP-32 protein. In a preferred embodiment of this type, the kit also contains a separate sample of phospho-Thr75 DARP-32 protein or fragment thereof to use as a standard. The kits can also comprise other reagents and/or written protocols.

Peptide Synthesis:

15 Synthetic polypeptides, can also be prepared using the well known techniques of solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and unnatural amino acids. Amino acids used for peptide synthesis may be standard Boc (N^{α} -amino protected N^{α} -t-butyloxycarbonyl) amino acid resin with the standard deprotecting, neutralization, coupling and wash protocols of the
 20 original solid phase procedure of Merrifield [*J. Am. Chem. Soc.*, **85**:2149-2154 (1963)], or the base-labile N^{α} -amino protected 9-fluorenylmethoxycarbonyl (Fmoc) amino acids first described by Carpino and Han [*J. Org. Chem.*, **37**:3403-3409 (1972)]. Both Fmoc and Boc N^{α} -amino protected amino acids can be obtained from The Rockefeller University Protein/DNA Technology Center, Fluka, Bachem, Advanced
 25 Chemtech, Sigma, Cambridge Research Biochemical, Bachem, or Peninsula Labs or other chemical companies familiar to those who practice this art. In addition, the method of the invention can be used with other N^{α} -protecting groups that are familiar to those skilled in this art. Solid phase peptide synthesis may be accomplished by techniques familiar to those in the art and provided, for example, in Stewart and
 30 Young, 1984, Solid Phase Synthesis, Second Edition, Pierce Chemical Co., Rockford,

IL; Fields and Noble, 1990, *Int. J. Pept. Protein Res.* **35**:161-214, or using automated synthesizers, such as sold by ABS. Thus, polypeptides of the invention may comprise D-amino acids, a combination of D- and L-amino acids, and various "designer" amino acids (*e.g.*, β -methyl amino acids, $C\alpha$ -methyl amino acids, and $N\alpha$ -methyl amino acids, etc.) to convey special properties. Synthetic amino acids include ornithine for lysine, fluorophenylalanine for phenylalanine, and norleucine for leucine or isoleucine. Additionally, by assigning specific amino acids at specific coupling steps, α -helices, β turns, β sheets, γ -turns, and cyclic peptides can be generated.

Traversing the Blood Brain Barrier:

- 10 Whereas the present invention includes the now standard (though fortunately infrequent) procedure of drilling a small hole in the skull to administer a drug of the present invention, in a preferred aspect, the agent/drug can cross the blood-brain barrier, which would allow for intravenous or oral administration. Many strategies are available for crossing the blood-brain barrier, including but not limited to, increasing
- 15 the hydrophobic nature of a molecule; introducing the molecule as a conjugate to a carrier, such as transferrin, targeted to a receptor in the blood-brain barrier, or to docosahexaenoic acid etc. In another embodiment, the molecule can be administered intracranially or, more preferably, intraventricularly. In another embodiment, osmotic disruption of the blood-brain barrier can be used to effect delivery of agent to the brain
- 20 [Nilaver *et al.*, *Proc. Natl. Acad. Sci. USA* **92**:9829-9833 (1995)]. In yet another embodiment, an agent can be administered in a liposome targeted to the blood-brain barrier. Administration of pharmaceutical agents in liposomes is known [*see* Langer, *Science* **249**:1527-1533 (1990); Treat *et al.*, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365
- 25 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*] All of such methods are envisioned in the present invention.

Although some predictions have been made concerning the ability of molecules to pass through the blood-brain barrier, these predictions are at best speculative. The rate and extent of entry of a compound into the brain are generally considered to be determined

primarily by partition coefficient, ionization constant(s), and molecular size. No single partition solvent system has emerged as a universally applicable model for brain penetration, although the octanol water system has received particular attention, and Hansch and coworkers have suggested that a partition coefficient in this system of

5 about 100 is optimal for entry into the central nervous system (CNS) [Glave and Hansch, *J. Pharm. Sci.* **61**:589 (1972); Hansch *et al.*, *J. Pharm. Sci.*, **76**:663 (1987)]. In practice, the octanol-water partition system only provides a qualitative indication of the capability of a compound to cross the blood-brain barrier. For example, comparisons between known histamine H₂ receptor antagonists suggest that there is no

10 such simple relationship between their brain penetration and octanol water partition coefficients [Young *et al.*, *J. Med. Chem.* **31**:656 (1988)]. Other factors, besides the octanol-water partition influence the propensity to cross the blood-brain barrier. Comparison of the ability of histamine H₂ receptor antagonists to cross the blood-brain barrier suggests that brain penetration may increase with decreasing over-all hydrogen

15 binding ability of a compound (Young *et al.*, *supra*). Begley *et al.* [*J. Neurochem.* **55**:1221-1230 (1990)] herein incorporated by reference in its entirety, have more recently examined the ability of cyclosporin A to cross the blood-brain barrier. Methodology as used by Begley *et al.* includes: (1) measuring the brain uptake index (BUI) with the equation for a tritiated agent compound:

20
$$\text{BUI} = [(\text{brain } ^3\text{H} / \text{brain } ^{14}\text{C}) / (\text{injectate } ^3\text{H} / \text{injectate } ^{14}\text{C})] \times 100$$
 where the ¹⁴C reference compound is ¹⁴C butanol or an analogous solvent;

(2) Brain perfusion studies; (3) Intravenous bolus injection studies; and (4) Studies with cultured cerebral capillary endothelium.

Labels:

25 Any of the potential agents and targets for the potential agents (*e.g.*, Cdk5) or DARPP-32 (such as ³²P-Thr75 phosphorylated DARPP-32) can be labeled. Suitable labels include enzymes, fluorophores (*e.g.*, fluoresceine isothiocyanate (FITC), phycoerythrin (PE), Texas red (TR), rhodamine, free or chelated lanthanide series salts, especially Eu³⁺, to name a few fluorophores), chromophores, radioisotopes, chelating agents,

30 dyes, colloidal gold, latex particles, ligands (*e.g.*, biotin), and chemiluminescent

In the instance where a radioactive label, such as the isotopes ^3H , ^{14}C , ^{32}P , ^{35}S , ^{36}Cl , ^{51}Cr , ^{57}Co , ^{58}Co , ^{59}Fe , ^{90}Y , ^{125}I , ^{131}I , and ^{186}Re are used, known currently available

Direct labels are one example of labels which can be used according to the present invention. A direct label has been defined as an entity, which in its natural state, is readily visible, either to the naked eye, or with the aid of an optical filter and/or applied stimulation, e.g. U.V. light to promote fluorescence. Among examples of colored labels, which can be used according to the present invention, include metallic sol particles, for example, gold sol particles such as those described by Leuvering (U.S. Patent 4,313,734); dye sol particles such as described by Gribnau *et al.* (U.S. Patent 4,373,932) and May *et al.* (WO 88/08534); dyed latex such as described by May, *supra*, Snyder (EP-A 0 280 559 and 0 281 327); or dyes encapsulated in liposomes as described by Campbell *et al.* (U.S. Patent 4,703,017). Other direct labels include a radionucleotide, a luminescent moiety, or a fluorescent moiety including as a modified/fusion chimera of green fluorescent protein (as described in U.S. Patent No. 5,625,048 filed April 29, 1997, and WO 97/26333, published July 24, 1997, the disclosures of each are hereby incorporated by reference herein in their entireties). In addition to these direct labelling devices, indirect labels comprising enzymes can also be used according to the present invention. Various types of enzyme linked immunoassays are well known in the art, for example, alkaline phosphatase and horseradish peroxidase, lysozyme, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, urease, these and others have been discussed in detail by Eva Engvall in Enzyme Immunoassay ELISA and EMIT in *Methods in Enzymology*, **70**. 419-439, 1980 and in U.S. Patent 4,857,453.

Suitable enzymes include, but are not limited to, alkaline phosphatase and horseradish peroxidase. Other labels for use in the invention include magnetic beads or magnetic resonance imaging labels.

- As exemplified herein, proteins can be labeled by metabolic labeling. Metabolic labeling occurs during *in vitro* incubation of the cells that express the protein in the presence of culture medium supplemented with a metabolic label, such as [³⁵S]-methionine or [³²P]-orthophosphate. In addition to metabolic (or biosynthetic) labeling with [³⁵S]-methionine, the invention further contemplates labeling with [¹⁴C]-amino acids and [³H]-amino acids (with the tritium substituted at non-labile positions).
- 10 The present invention also provides fusion proteins comprising the phospho-Thr75 DARPP-32 protein and fragments thereof which have been "modified" *i.e.*, placed in a fusion of chimeric peptide or protein, or labeled, *e.g.*, to have an N-terminal FLAG-tag, or a C-terminal 6X His-tag. In a particular embodiment a phospho-Thr75 DARPP-32 fragment or full-length protein is modified to contain a marker protein such as green fluorescent protein as described in U.S. Patent No. 5,625,048 filed April 29, 1997 and WO 97/26333, published July 24, 1997 (each of which are hereby incorporated by reference herein in their entireties), glutathione-S-transferase (GST) as described in Example 1 below, or a poly-histidine tag.

20 General Genetic Manipulations

- The present invention also provides methods of expressing specific drug targets, kinases, and/or kinase substrates including vectors containing nucleic acids encoding DARPP-32 and analogs and derivatives of DARPP-32. Included are homologs of human and mouse DARPP-32 and fragments thereof, from other species. Therefore the production and use of derivatives and analogs related to DARPP-32 are within the scope of the present invention.

DARPP-32 derivatives, for example, can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions including to provide for functionally

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equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a DARPP-32 gene may be used in the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, and

5 nucleotide sequences comprising all or portions of DARPP-32 genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the DARPP-32 derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a DARPP-32 including altered

10 sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may

15 be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and

20 glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred substitutions are:

- 25 - Lys for Arg and vice versa such that a positive charge may be maintained;
 - Glu for Asp and vice versa such that a negative charge may be maintained;
 - Ser for Thr such that a free -OH can be maintained; and
 - Gln for Asn such that a free NH₂ can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced at a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (*i.e.*, His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β -turns in the protein's structure.

The genes encoding drug targets, kinases, kinase substrates such as DARPP-32 derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned DARPP-32 gene sequence can be modified by any of numerous strategies known in the art [Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York]. The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of DARPP-32, care should be taken to ensure that the modified gene remains within the same translational reading frame as the DARPP-32 gene, uninterrupted by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the DARPP-32-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated DARPP-32 gene product.

Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis [Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710], use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis

[see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70].

A nucleotide sequence coding for DARPP-32, a fragment of DARPP-32 or a derivative or analog thereof, including a functionally active derivative, such as a chimeric protein, thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding a DARPP-32 of the invention or a fragment thereof is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

The necessary transcriptional and translational signals can be provided on a
15 recombinant expression vector, or they may be supplied by the native gene encoding
DARPP-32 and/or its flanking regions.

Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

A recombinant DARPP-32 protein of the invention, or DARPP-32 fragment, derivative, chimeric construct, or analog thereof, may be expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a

number of amplification systems may be used to achieve high levels of stable gene expression [See Sambrook et al., 1989, *supra*].

A cell containing the recombinant vector comprising the nucleic acid encoding DARPP-32 for example, can be cultured in an appropriate cell culture medium under conditions that provide for expression of DARPP-32 by the cell.

DNA fragments can be readily inserted into an expression vector consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

10 Expression of DARPP-32 may be controlled by any promoter/enhancer element known
in the art, but these regulatory elements must be functional in the host selected for
expression. Promoters that may be used to control DARPP-32 gene expression are
well known in the art including prokaryotic expression vectors such as the β -lactamase
promoter [Villa-Kamaroff, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **75**:3727-3731 (1978)],
15 or the *tac* promoter [DeBoer, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, **80**:21-25 (1983)].

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMal-C2, pET, pGEX [Smith *et al.*, *Gene*, 67:31-40 (1988)], pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage λ , e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 μ plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

(*Bam*H1, *Sma*I, *Xba*I, *Eco*R1, *Not*I, *Xma*III, *Bg*III, and *Pst*I cloning site; Invitrogen), pVL1392 (*Bg*III, *Pst*I, *Not*I, *Xma*III, *Eco*RI, *Xba*I, *Sma*I, and *Bam*H1 cloning site;

- 5 Summers and Invitrogen), and pBlueBacIII (*Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, with blue/white recombinant screening possible; Invitrogen), and fusion transfer vectors, such as but not limited to pAc700 (*Bam*H1 and *Kpn*I cloning site, in which the *Bam*H1 recognition site begins with the initiation codon; Summers), pAc701 and pAc702 (same as pAc700, with different reading frames), pAc360 (*Bam*H1 cloning site 36 base pairs downstream of a polyhedrin initiation codon; Invitrogen(195)), and pBlueBacHisA, B, C (three different reading frames, with *Bam*H1, *Bgl*II, *Pst*I, *Nco*I, and *Hind*III cloning site, an N-terminal peptide for ProBond purification, and blue/white recombinant screening of plaques; Invitrogen (220)) can be used.
- 15 Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current*
- 20 *Protocols in Molecular Biology*, 16.12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be
- 25 used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker;
- 30

- Invitrogen), pREP8 (*Bam*HI, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and *Bam*HI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen).
- 5 Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection; Invitrogen), and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman, 1991,
- 10 *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and β -gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*I, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and β -gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and *Hpa*I cloning site, TK or XPRT selection).
- 15 Yeast expression systems can also be used according to the invention to express the bacterial DARPP-32. For example, the non-fusion pYES2 vector (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Gst*XI, *Eco*RI, *Bst*XI, *Bam*HI, *Sac*I, *Kpn*I, and *Hind*III cloning sit; Invitrogen) or the fusion pYESHisA, B, C (*Xba*I, *Sph*I, *Sho*I, *Not*I, *Bst*XI, *Eco*RI, *Bam*HI, *Sac*I, *Kpn*I, and *Hind*III cloning site, N-terminal peptide purified with ProBond resin and cleaved
- 20 with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated

25 and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

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Vectors can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter [see, *e.g.*, Wu *et al.*, *J. Biol. Chem.*, **267**:963-967 (1992); Wu and Wu, *J. Biol. Chem.*, **263**:14621-14624 (1988); Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Phosphorylation State-specific Antibodies Having Specificity
for Thr75-phosphorylated DARPP-32

According to the present invention, the Thr75-phosphorylated DARPP-32 or fragment containing Threonine-75 as produced by a recombinant source, or through chemical synthesis, proteolysis of the DARPP-32 or as isolated from natural sources; and derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize Thr75-phosphorylated DARPP-32 as exemplified below. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and a Fab expression library. The Thr75-phosphorylated DARPP-32 antibodies of the invention may be cross reactive, that is, they may recognize a Thr75-phosphorylated DARPP-32 derived from a different source. Polyclonal antibodies have greater likelihood of cross reactivity. Alternatively, an antibody of the invention is preferably specific for the Thr75-phosphorylated DARPP-32 having an amino acid sequence of SEQ ID NO:1.

Various procedures known in the art may be used for the production of polyclonal antibodies to Thr75-phosphorylated DARPP-32 or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the Thr75-phosphorylated DARPP-32, or a derivative (*e.g.*, or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the Thr75-phosphorylated DARPP-32 can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels

5 Fab fragments with the desired specificity for Thr75-phosphorylated DARPP-32.

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25 antibodies which recognize a specific epitope of Thr75-phosphorylated DARPP-32,

one may assay generated hybridomas for a product which binds to the Thr75-phosphorylated DARPP-32 fragment containing such epitope and choose those which do not cross-react with Thr75-phosphorylated DARPP-32. For selection of an antibody specific to a Thr75-phosphorylated DARPP-32 from a particular source, one

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the Thr75-phosphorylated DARPP-32, *e.g.*, for Western blotting, imaging Thr75-phosphorylated DARPP-32 *in situ*, measuring levels thereof in appropriate physiological samples, etc. using any of the detection techniques mentioned herein or known in the art.

The particular steps involved in the generation of phosphorylation state-specific antibodies (PSSAb) to Thr75 DARPP-32 exemplified below are shown in Figures 6A-10 6F [see Czernik *et al.*, in *Regulatory Protein Modification* (ed., H.C. Hemming, Jr) Pg. 219-246 (Humana, Tototwa NJ (1997) hereby incorporated by reference in its entirety]. The particular phospho-peptide used to generate the P-Thr75 DARPP-32 phosphorylation state-specific antibody used in Example 1 below has the amino acid sequence of CAYTPPSLK, in which the threonine residue was chemically 15 phosphorylated. Thus a selected phospho-peptide that corresponds to a fragment of the phosphoprotein of interest containing the particular phosphorylated amino acid residue of interest can be prepared (*e.g.*, by peptide synthesis). The phospho-peptide is preferably conjugated to a carrier protein using an appropriate crosslinker and then inoculated into a non-human animal subject (*e.g.*, a sheep or a rabbit). The inoculated 20 non-human animal can then be boosted and bled as required to obtain sera containing the desired antibody. Primary screening (*e.g.*, by immunoblotting) of the antisera is performed to identify an antibody that is specific for the particular phosphoprotein (*i.e.*, phospho-Thr75 DARPP-32). Preferably, the phospho-specific antibody is then purified. One such method includes using Protein A and phospho/dephosphopeptide 25 affinity chromatography [see Czernik *et al.*, in *Regulatory Protein Modification* (ed., H.C. Hemming, Jr) Pg. 219-246 (Humana, Tototwa NJ (1997))].

Administration

According to the invention, the component or components of a therapeutic composition of the invention may be introduced parenterally, topically, or transmucosally, *e.g.*, orally, nasally, or rectally, or transdermally. Preferably, administration is parenteral,
 5 *e.g.*, via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration.

In another embodiment, the therapeutic compound can be delivered in a vesicle, in particular a liposome [*see* Langer, *Science* **249**:1527-1533 (1990); Treat *et al.*, in
 10 *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss: New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*]. To reduce its systemic side effects, this may be a preferred method for introducing the agent.

In yet another embodiment, the therapeutic compound can be delivered in a controlled
 15 release system. For example, the agent may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used [*see* Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* **14**:201 (1987); Buchwald *et al.*, *Surgery* **88**:507 (1980); Saudek *et al.*, *N. Engl. J. Med.* **321**:574 (1989)]. In another embodiment,
 20 polymeric materials can be used [*see Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Press: Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley: New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* **23**:61 (1983); *see* also Levy *et al.*, *Science* **228**:190 (1985); During *et al.*, *Ann. Neurol.* **25**:351 (1989); Howard *et al.*, *J. Neurosurg.* **71**:105 (1989)]. In yet another
 25 embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose [*see, e.g.*, Goodson, in *Medical Applications of Controlled Release, supra*, vol. 2, pp. 115-138 (1984)].

Other controlled release systems are discussed in the review by Langer [*Science* 249:1527-1533 (1990)].

Pharmaceutical Compositions: In yet another aspect of the present invention, provided are pharmaceutical compositions of the above. Such pharmaceutical compositions may be for administration for injection, or for oral, topological, nasal or other forms of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of the agents of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hyaluronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilized form.

Oral Delivery: Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (*e.g.*, U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall,

K. In: *Modern Pharmaceu***t***ics* Edited by G.S. Banker and C.T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include the agent and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

- 5 To ensure full gastric resistance a coating impermeable to at least pH 5.0 is useful. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and
10 Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic i.e. powder; for liquid forms, a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The therapeutic can be included in the formulation as fine multi-particulates in the form of granules or pellets of particle size about 1 mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs or even as tablets. The therapeutic could be prepared by compression.

Colorants and flavoring agents may all be included. For example, the protein (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the therapeutic with an inert material. These diluents could include carbohydrates, especially mannitol, α -lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrates include but are not limited to starch, including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. Another form of the disintegrants are the insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders and these can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the therapeutic agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the therapeutic.

An anti-frictional agent may be included in the formulation of the therapeutic to prevent sticking during the formulation process. Lubricants may be used as a layer between the therapeutic and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the therapeutic into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethonium chloride. The list of potential non-ionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the agent are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release oral formulation may be desirable. The agent could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms, *e.g.*, gums. Slowly degenerating matrices may also be incorporated into the formulation. Some enteric coatings also have a delayed release effect.

Another form of a controlled release of this therapeutic is by a method based on the Oros therapeutic system (Alza Corp.), *i.e.* the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The therapeutic agent could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The

first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are
 5 commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan-coater or in a fluidized bed or by compression coating.

Nasal Delivery. Nasal delivery of the agent is also contemplated. Nasal delivery
 10 allows the passage of the protein to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

Methods of Treatment, Methods of Preparing a Medicament: In yet another aspect of the present invention, methods of treatment and manufacture of a medicament are
 15 provided. Conditions alleviated or modulated by the administration of the present derivatives are those indicated above.

Dosages. For any of the agents, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context,
 20 age and general health of the recipient, will be able to ascertain proper dosing. Generally, for intravenous injection or infusion, dosage may be lower. The dosing schedule may vary, depending on the circulation half-life, and the formulation used.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following
 25 examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

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EXAMPLE 1Cdk5 PHOSPHORYLATION OF DARPP-32
MODULATES DOPAMINE SIGNALING IN NEURONSIntroduction

5 Numerous first messengers modulate the phosphorylation and dephosphorylation of DARPP-32 at Threonine-34 in intact cells, thereby altering PP1 activity and regulating the states of phosphorylation and activities of a variety of downstream physiological effectors. The biological significance of this pathway has been demonstrated using mice containing a targeted deletion of the DARPP-32 gene (DARPP-32^{-/-}), which

10 exhibit a dramatically altered biochemical, electrophysiological, and behavioral phenotype [U.S. Patent No:5,777,195, Issued July 7, 1998; Fienberg *et al.*, *Science* **281**:838-842 (1998), the contents of each are hereby incorporated by reference herein, in their entireties]. The amino acid sequence of DARPP-32 also contains consensus phosphorylation sites for proline-directed kinases including Cdk5, a cyclin-dependent

15 kinase family member, that together with its non-cyclin cofactor, Nck5a (p35), is present in post-mitotic neurons expressing high levels of DARPP-32 [Lew *et al.*, *Nature* **371**:423-426 (1994); Tsai *et al.* *Nature* **371**:419-423 (1994)].

Results

Cdk5, immunoprecipitated from mouse striatal homogenates, phosphorylated purified

20 DARPP-32 *in vitro* (Figs. 1a and 1b). Phosphorylation was greatly reduced by the addition of the specific Cdk5 inhibitor roscovitine [Meijer *et al.*, *Eur. J. Biochem.* **243**:527-536 (1997)]. DARPP-32 was much less efficiently phosphorylated by Cdk5 immunoprecipitated from striatal homogenates of p35^{-/-} (Nck5a^{-/-}) mice (Fig. 1b).

Purified DARPP-32 was phosphorylated *in vitro* by recombinant GST-Cdk5 (Fig. 1c)

25 as well as by recombinant cdk1. Proteolytic digestion, HPLC purification, phosphopeptide mapping, microsequencing, and MALDI-TOF mass spectrometry analysis indicated that the site of phosphorylation by either Cdk5 or cdk1 was Threonine-75 (Thr75). A recombinant protein containing a Thr75Ala mutation was not phosphorylated, indicating that Thr75 is the only site phosphorylated by Cdk5 and

cdk1. In contrast, MAP kinase, another proline-directed kinase, did not phosphorylate DARPP-32 at Thr75.

To directly monitor DARPP-32 phosphorylation by Cdk5 *in vitro* and in brain tissue, a phosphorylation state-specific antibody was generated which detected DARPP-32 only in its Thr75 phosphorylated state (Fig. 1c). DARPP-32 was phosphorylated at Thr75 in freshly prepared adult mouse striatal tissue (Figs. 1d and 1e). Comparison of phospho-Thr75 and total DARPP-32 standards indicated that the stoichiometry of Thr75 DARPP-32 phosphorylation was ~0.26 under basal conditions. The level of DARPP-32 in striatal neurons has been estimated to be ~50 μ M [Ouimet *et al.*, *Brain Res.* **808**:8-12 (1998)]. Therefore, the basal concentration of phospho-Thr75 DARPP-32 was ~13 μ M. The basal level of phospho-Thr75 DARPP-32 was reduced by 75% in p35^{-/-} mouse striatum, whereas the total level of DARPP-32 was unaffected (compare Fig. 1d with Fig. 1e).

The role of DARPP-32 phosphorylated at Thr75 was assessed in striatal slices from adult mice. Inhibition of Cdk5 by roscovitine treatment reduced phospho-Thr75 DARPP-32 without affecting the total level of DARPP-32 (Figs. 2a and 2b). Remarkably, roscovitine treatment increased phospho-Thr34 DARPP-32 (Fig 2c). The results suggested a reciprocal relationship between the two phosphorylation sites. A biochemical approach was used to determine the mechanism underlying this relationship. The ability of PKA to phosphorylate Thr34 DARPP-32 *in vitro* was virtually abolished by prior phosphorylation at Thr75 (Figs. 2d-2f). To test the possibility that this effect was due to phospho-Thr75 acting as an inhibitor of PKA, the ability of the purified catalytic subunit of PKA to phosphorylate several other well-characterized substrates was assessed in the absence and presence of added phospho-Thr75 DARPP-32. The presence of 15 μ M phospho-Thr75 DARPP-32 resulted in a dramatic reduction in the rates of phosphorylation by PKA of inhibitor-1 (a DARPP-32 homologue), ARPP-21 (a PKA substrate enriched in the basal ganglia [Hemmings *et al.*, *J. Biol. Chem.* **264**:7726-7733 (1989)], synapsin I, and histone H1. Kinetic analysis of ARPP-21 phosphorylation in the presence of various concentrations

of phospho-Thr75 DARPP-32 indicated that phospho-Thr75 DARPP-32 inhibited PKA by a linear competitive single site mechanism (Fig. 2g). A K_i value of $2.7 \mu\text{M}$ was derived from a secondary plot of the kinetic data (Fig. 2g, insert).

To determine whether phospho-Thr75 DARPP-32 inhibits PKA in intact neurons, phosphorylation of known substrates was assessed in mouse striatal slices treated with roscovitine to inhibit Cdk5. Roscovitine increased phosphorylation of the GluR1 subunit of the AMPA-type glutamate receptor by 3.2 ± 0.5 fold ($n = 7$), as assessed using an antibody specific for phospho-Ser845, a PKA phosphorylation site [Roche *et al.*, *Neuron* **16**:1179-1188 (1996)] (Fig. 3a). A roscovitine-induced increase in phospho-Ser845 GluR1 was not observed in striatal slices from adult DARPP-32^{-/-} mice [U.S. Patent No:5,777,195, Issued July 7, 1998; Fienberg *et al.*, *Science* **281**:838-842 (1998)], providing strong support for the involvement of phospho-Thr75 in this action of roscovitine. Total GluR1 levels were unaffected. Similar results (2.5 ± 0.2 fold, $n = 2$) for wildtype and DARPP-32^{-/-} mice were observed for phosphorylation of ARPP-16 (Fig.3b), a PKA substrate enriched in striatum [Horiuchi *et al.*, *J. Biol. Chem.* **265**:9476-9484 (1990)]. These results indicate that regulation by Cdk5 of the phosphorylation of Thr75 DARPP-32 has a major effect on PKA activity in intact cells.

Dopamine achieves many of its effects in neurons by activation of the D1 class of dopamine receptors, which are positively coupled to the activation of PKA. To determine whether phospho-Thr75 DARPP-32 modulates the actions of dopamine in intact cells, the ability of a D1 receptor agonist (SKF 81297) to induce phosphorylation of substrates for PKA was compared in wildtype and p35^{-/-} mice. For this purpose, the state of phosphorylation of GluR1, DARPP-32, ARPP-21, and ARPP-16 was determined using phosphorylation state-specific antibodies. SKF 81297 increased the phosphorylation of each of these substrates in the wildtype mice, and did so to an even greater extent in the p35^{-/-} mice (Figs. 3c-3f). No significant difference was observed between wildtype and p35^{-/-} mice in basal levels of phosphorylation of any of these substrates. These data indicate that Cdk5 phosphorylation of DARPP-32 at Thr75 is capable of modulating dopamine signaling in the striatum.

The physiological consequence of PKA inhibition by phospho-Thr75 DARPP-32 was also assessed. For this purpose, voltage-gated Ca^{2+} currents, which are known to be regulated by PKA [Surmeier *et al.*, *Neuron* **14**:385-397 (1995); Gray *et al.*, *Curr. Opin. Neurobiol.* **8**:330-334 (1998)], were analyzed using patch-clamp recordings of

- 5 dissociated striatal neurons (Figs. 3g and 3h). Application of roscovitine ($10 \mu\text{M}$) enhanced whole-cell Ca^{2+} current in wildtype neurons ($9.7\% \pm 1.0\%$, $n = 7$). This effect was virtually abolished in neurons from DARPP-32 knockout mice ($1.6 \pm 0.3\%$, $n = 5$, $p < 0.01$, paired t test). These data indicate that regulation of the state of phosphorylation of Thr75 DARPP-32 can modulate the physiological state of neurons
10 in the striatum.

- The specificity of intracellular signaling can be controlled by scaffolding and anchoring molecules so that kinases as well as phosphatases are localized to their substrates [Pawson *et al.*, *Science*, **278**:2075-2080 (1997)]. Moreover, protein kinases and protein phosphatases can directly associate in signaling modules [Westphal *et al.*,
15 *J. Biol. Chem.* **274**:689-692 (1999); Klauck, *Science* **271**:1589-1592 (1996)]. The data presented here show that, through phosphorylation of distinct sites, DARPP-32 can regulate both classes of activity (Fig. 4). This dual action appears especially important in regulating the efficacy of dopaminergic neurotransmission. These results also indicate a novel role for Cdk5 in the control of the actions of dopamine. The
20 ability of one protein to regulate both a major protein kinase and a major protein phosphatase represents a new mechanism by which cell signaling pathways may be integrated.

EXAMPLE 2CDK5 REGULATES ACTION OF CHRONIC COCAINEIntroduction

Cocaine, is a psychomotor stimulant extracted from the native South American plant, *Erythroxylon coca*, that was introduced to the general public as a therapeutic agent by the medical community in the late 19th century [Freud *et al.*, *Centralblatt für die Gesellschaft Therapie* **2**:289-314 (1884); reprinted in English, I. *Subst. Abuse Treatment*, 206-217 (1984)]. Widespread cocaine abuse today represents a major world public health problem having a serious overall impact on society [Higgins and Katz, *Cocaine Abuse: Behavior, Pharmacology, and Clinical Applications* (Academic Press, New York, 1998)]. Cocaine enhances dopamine neurotransmission by blocking dopamine reuptake at axon terminals innervating medium spiny neurons in the caudatoputamen and nucleus accumbens, which together comprise the striatum. Cocaine addiction is thought to stem, in part, from neural adaptations that act to maintain equilibrium by countering the effects of repeated drug administration [Nestler and Aghajanian, *Science* **278**:58-63 (1997); Berke and Hyman, *Neuron* **25**:515-532 (2000); Koob and Le Moal, *Science* **278**:52-57 (1997)]. Chronic exposure to cocaine upregulates several transcription factors that alter gene expression and could mediate such compensatory neural and behavioral plasticity [Kelz *et al.*, *Nature* **401**:272-276 (1999); Nestler *et al.*, *Brain Res.* **835**:10-17 (1999); Hope *et al.*, *Neuron* **13**:1235-1244 (1994); Carlezon *et al.*, *Science* **282**:2272-2275 (1998)]. One such transcription factor is Δ FosB, a highly stable protein that persists in striatum long after cessation of cocaine exposure [Hiroi *et al.*, *Proc. Natl. Acad. Sci. USA* **94**:10397-10402 (1997)]. Mice with altered Δ FosB expression exhibit abnormal behavioral responses to cocaine [Kelz *et al.*, *Nature* **401**:272-276 (1999); Hiroi *et al.*, *Proc. Natl. Acad. Sci. USA* **94**:10397-10402 (1997)].

Methods

For cDNA expression arrays, total RNA was isolated with the RNAqueous phenol-free total RNA isolation kit (Ambion) from dissected striatal tissue from either control mice

carrying only the NSE-tTA gene ($-\Delta\text{FosB}$) or ΔFosB -inducible transgenic mice carrying both Tetop- ΔFosB and NSE-tTA genes, which had been fed 100 mg/l doxycycline to inhibit transgene expression, followed by 12 weeks in the absence of doxycycline ($+\Delta\text{FosB}$). ΔFosB induction was observed by 3 weeks [Chen *et al.*, *Mol. Pharmacol.* 54:495-503 (1998)]. Poly(A)+ RNA was isolated from total RNA using an Oligotex mRNA isolation kit (Qiagen) and used as template for synthesis of ^{32}P -labeled cDNA probes. The probes were hybridized to Atlas cDNA expression arrays containing 588 genes (Clontech). Probes were hybridized and chips were washed according to the manufacturer's suggestions. Radiographic images were generated using a Storm PhosphorImager (Molecular Dynamics).

ΔFosB -inducible transgenic mice were either fed 100 mg/l doxycycline or denied doxycycline for 12 weeks. Adult male Sprague-Dawley rats initially weighing between 160-240 g were injected with cocaine or vehicle (i.p., 20 mg/kg, 9% NaCl) at the same time each day for 8 days. All analyses were initiated 12 hr after the final dose. For *in situ* hybridization studies, [α - ^{35}S]UTP-labeled riboprobes were prepared by *in vitro* transcription from cDNA clones corresponding to full-length clones of rat Cdk5 [Zheng *et al.*, *J. Neurobiol.* 35:141-159 (1998)]. Cryostat sections were prepared and hybridized as previously described. After hybridization, the sections were exposed to Biomax MR film (Kodak) for 2-6 days. All autoradiograms were analyzed with a Microcomputer Imaging Device system (M4, Imaging Research, Inc.) as described elsewhere [Le Moine and Bloch, *J. Comp. Neurol.* 355:418-426 (1995)]. Statistical analyses of the data were performed using two-tailed unpaired Student's *t* test. Striatal tissue was rapidly dissected on ice and homogenized in boiling lysis buffer containing 1% SDS and 50 mM sodium flouride with sonication. Striatal slice preparation and treatment were conducted as previously described [Example 1, Bibb *et al.*, *Nature* 402:669-671 (1999); Nishi *et al.*, *J. Neurosci* 17:8147-8155 (1997)]. Use of antibodies to phospho-Thr34 DARPP-32 [Snyder *et al.*, *J. Neurosci* 12:3071-3083 (1992)], phospho-Thr75 DARPP-32 [Example 1, Bibb *et al.*, *Nature* 402:669-671 (1999)], phospho-Ser55 ARPP-21 [Caporaso *et al.*, *Neuropharmacology* 39:1637-1644 (1999)], phospho-Ser88 ARPP-16 [Example 1, Bibb *et al.*, *Nature* 402:669-671 (1999)], and

phospho-Ser845 GluR1 [Lee *et al.*, *Neuron* 21:1151-1162 (1998)] for immunoblotting was performed as previously described.

- Drug-induced alterations in cocaine sensitization were measured in adult male Sprague-Dawley rats according to published procedures for surgery, drug infusion, apparatus, and behavioral methods [Horger *et al.*, *J. Neuroscience* 19:410-4155 (1999); Taylor and Horger, *Psychopharmacology* 142:31-40 (1999)]. Coordinates for the nucleus accumbens were anterior-posterior 1.7 from bregma, medial-lateral \pm 1.5 from the midline, dorso-ventral - 6.0 from skull [Paxinos and Watson, *The rat brain in stereotaxic coordinates* (Academic Press, New York, 1982)].
- 10 Doses of cocaine hydrochloride (15 mg/kg i.p. in sterile 0.9% sodium chloride) were chosen to produce a moderate response to cocaine over successive days. Roscovitine, olomoucine, and iso-olomoucine (Alexis Biochemicals) doses (40 nmol /0.5 μ l) were based on previous studies using intra-cerebral infusions of cAMP analogs [Punch *et al.*, *J. Neurosci.* 17:8520-8527 (1997)]. Drugs were dissolved in sterile phosphate
- 15 buffered saline containing 50% DMSO. Intracerebral microinfusions were made bilaterally in volumes of 0.5 μ l over a period of 2 min with an additional 2 min allowed to elapse prior to removal of the infusion needles and replacement of the stylettes. One week after surgery and 2 days after habituation to the locomotor chambers, subjects received 5 daily injections of cocaine (15 mg/kg, i.p.) or saline 20
- 20 min after bilateral intra-accumbens vehicle, roscovitine, olomoucine, or iso-olomoucine infusions. Drugs were given at the same time each day. Subjects were then placed into the chambers and activity was monitored for 60 min.

- For electrophysiological studies [Surmeier *et al.*, *Neuron* 14:385-397 (1995)], acutely dissociated striatal neurons were prepared as previously described. Whole-cell
- 25 recordings of voltage-gated AMPA/kainate-activated current (100 μ M kainate) were measured using standard whole-cell voltage-clamp techniques [Hamill *et al.*, *Pflüegers Arch.* 391:85-100 (1981)]. Summary data are presented in box plot format [J. W. Tukey, *Exploratory Data Analysis* (Addison-Wesley, Menlo Park, CA, 1977)].

Results

Transgenic mice displaying inducible and targeted expression of Δ FosB in the nucleus accumbens and caudatoputamen were engineered [Chen *et al.*, *Mol. Pharmacol.* 54:495-503 (1998), *see* Methods above]. Analysis of cDNA expression array profiles from mice overexpressing Δ FosB indicated that the neuronal protein kinase Cdk5 was a downstream target gene for Δ FosB in these brain regions (Fig. 8A). This effect was confirmed by quantitative *in situ* hybridization analyses of coronal brain sections from mice that did (H₂O) or did not (Dox) overexpress Δ FosB (Fig. 8B, left). Increased Cdk5 mRNA expression in response to Δ FosB accumulation was evident in both the caudatoputamen ($160.0 \pm 14.2\%$, $p < 0.05$) and the nucleus accumbens ($152.6 \pm 12.9\%$, $p < 0.05$). Adult rats injected with cocaine for 8 days also showed elevated Cdk5 mRNA levels in the caudatoputamen ($151.8 \pm 11.8\%$, $p < 0.05$) and nucleus accumbens ($150.5 \pm 10.0\%$, $p < 0.05$) in comparison to animals injected with saline (Fig. 8B). Increased Cdk5 protein levels were also observed in striatal tissue dissected from transgenic Δ FosB-expressing mice ($130 \pm 10\%$, $p < 0.05$) and from rats exposed chronically to cocaine ($180 \pm 20\%$, $p < 0.05$) compared to control animals (Fig. 8C). These results indicated that Cdk5 is a downstream target of chronic cocaine exposure and raised the possibility that this protein kinase is involved in the behavioral effects of cocaine.

A characteristic behavioral effect of cocaine is potentiation of locomotor activity. To determine the possible contribution of elevated Cdk5 levels to long-lasting alterations in this behavior, the effects of daily intra-accumbens infusions of a potent Cdk5 inhibitor, roscovitine [Meijer *et al.*, *Eur. J. Biochem.* 243:527-536 (1997)], on cocaine-induced locomotor activity were examined over a 60 min period daily for five days. Roscovitine infusions did not significantly affect locomotor responses to initial cocaine administration. However, roscovitine markedly potentiated the locomotor effects of repeated cocaine exposures. This was evident as an augmentation of cocaine's effects over successive days of injections (Fig. 9A). By day 4, significant differences were observed between the saline/cocaine and roscovitine/cocaine groups. By day 5, mean cocaine-induced activity rates for roscovitine-infused animals were

almost double those measured for vehicle-infused animals. This effect of roscovitine was most evident at 40-60 min after cocaine administration (Fig. 9B), indicating that roscovitine prolonged cocaine-induced increases in locomotor activity.

Repeated intra-accumbens infusions of a less selective Cdk5 inhibitor, olomoucine
 5 [Abraham *et al.*, *Biol. Cell* 83:105-120 (1997)], also potentiated cocaine's locomotor effects. This action was similar to that produced by roscovitine, except that a dramatic behavioral effect was already observed on day 3 (Fig. 9C). In response to drug treatment on days 4 and 5, olomoucine-treated animals exhibited stereotypy, which is known to compete with increases in locomotor activity, [see Hiroi *et al.*, *Proc. Natl.*
 10 *Acad. Sci. USA* 94:10397-10402 (1997)]; these results are consistent with enhanced sensitization to repeated doses of cocaine in the presence of a Cdk5 inhibitor. In contrast, intra-accumbens infusions of the inactive congener, iso-olomoucine, failed to enhance either locomotor or stereotypic responses to cocaine (Fig. 9C). These behavioral findings indicate that cocaine-induced increases in Cdk5 levels may serve a
 15 homeostatic function to dampen responses to subsequent drug exposure.

One way by which Cdk5 could downregulate the psychomotor effects of chronic cocaine is through regulation of dopamine signaling. Cdk5 phosphorylates a key molecule involved in striatal dopamine signaling, DARPP-32 (Dopamine and cyclic AMP-Regulated Phospho-Protein, Mr 32 kDa), at threonine-75 (Thr75) [Example 1,
 20 Bibb *et al.*, *Nature* 402:669-671 (1999)]. Therefore, the possibility was examined as to whether the increases in Cdk5 levels brought about either by Δ FosB overexpression in mice or by chronic exposure to cocaine in rats. Thus, these increases in Cdk5 levels could be reflected in alterations in the state of phosphorylation of DARPP-32 at Thr75. Increased levels of phospho-Thr75 DARPP-32 were indeed observed in striatal tissue
 25 dissected from transgenic mice overexpressing Δ FosB (Fig. 10A). In rats subjected to chronic cocaine administration the levels of phospho-Thr75 DARPP-32 were increased in both the caudatoputamen and nucleus accumbens (Fig. 10B and 10C). In contrast, total levels of DARPP-32 were unaffected by these treatments.

Cdk5-dependent phosphorylation of DARPP-32 at Thr75 reduces the efficacy of dopamine/PKA/DARPP-32/PP-1 signaling [Example 1, Bibb *et al.*, *Nature* 402:669-671 (1999)]. To further test the possibility that the chronic cocaine-dependent regulation of Cdk5 activity was biologically relevant, various aspects of this signaling pathway were examined. Treatment of striatal slices from saline-injected rats with the D₁ receptor agonist, SKF 81297, is known to increase PKA-dependent phosphorylation of DARPP-32 [Snyder *et al.*, *J. Neurosci* 12:3071-3083 (1992)], ARPP-16 [Dulubova *et al.*, *J. Neurochem.* (submitted) (2000)], and ARPP-21 [Caporaso *et al.*, *Neuropharmacology* 39:1637-1644 (1999)]. These effects of the D₁ agonist were abolished in striatal slices from rats exposed to chronic cocaine (Fig. 11A). Decreased PKA-dependent phosphorylation of DARPP-32 (at Thr34) and of the GluR1 subunit of the AMPA-type glutamate receptor (at Ser845) was observed in striatal tissue dissected from rats chronically treated with cocaine (Fig. 11B). Consistent with the ability of PKA-dependent phosphorylation of GluR1 to increase AMPA channel conductance [Roche *et al.*, *Neuron* 16:1179-1188 (1996)], reduced peak amplitudes of AMPA-evoked Ca²⁺ currents in striatal neurons from chronic cocaine-treated rats were observed (Fig. 11C). These results expand upon the observation that nucleus accumbens neurons from psychomotor stimulant-treated rats were less sensitive to the rate-enhancing effects of glutamate [White *et al.*, *J. Pharmacol. Exp. Ther.* 273:445-454 (1995)].

While total levels of PKA catalytic subunit are increased in the nucleus accumbens after chronic cocaine administration [Nestler and Aghajanian, *Science* 278:58-63 (1997); Self and Nestler, *Annu. Rev. Neurosci.* 18:463-495 (1995)], the level of PKA activity within accumbens neurons in the intact brain has not been previously assessed. The results presented here indicate that chronic exposure to cocaine is, in fact, associated with a decrease in PKA activity, as evidenced by a reduction in the ability of a D₁ agonist to stimulate PKA-dependent phosphorylation of DARPP-32, ARPP-16, and ARPP-21, by lowered basal phosphorylation of DARPP-32 (at Thr34) and GluR1 (at Ser845), and by decreased conductance of the AMPA channel. The attenuation of PKA-dependent signaling by chronic cocaine can be explained by increased

Cdk5-dependent phosphorylation of DARPP-32 on Thr75. Viewed in this way, the increase in total PKA levels observed in earlier studies represents a compensatory change secondary to the decrease in PKA activity.

There is now considerable evidence that the D1 receptor plays an important role in mediating the behavioral effects of cocaine [White *et al.*, *J. Pharmacol. Exp. Ther.* 273:445-454 (1995); Self and Nestler, *Annu. Rev. Neurosci.* 18:463-495 (1995); Dreher and Jackson, *Brain Res.* 487:267-277 (1989); Xu *et al.*, *Cell* 79:945-955 (1994); Henry and White, *J. Pharmacol. Exp. Ther.* 258:882-890 (1991)]. Taken together, the data reported here support a biochemical scheme in which repeated exposure to cocaine causes accumulation of Δ FosB, which in turn results in increased expression of Cdk5; increased Cdk5-dependent phosphorylation of DARPP-32 at Thr75 then reduces PKA activity and attenuates D₁-dopamine receptor signaling. In support of the behavioral relevance of this scheme, mice lacking the DARPP-32 gene [Fienberg *et al.*, *Science* 281:838-842 (1998); Hiroi *et al.*, *Eur. J. Neurosci.* 11:1114-1118 (1999)] and rats treated with Cdk5 inhibitors (Fig. 9) both exhibit enhanced behavioral responses to chronic administration of cocaine. The ability of intra-accumbens infusions of Cdk5 inhibitors to potentiate the locomotor effects of repeated cocaine administration suggests that the Cdk5/DARPP-32 pathway serves a negative feedback homeostatic role with respect to the behavioral effects of cocaine. Other actions of Cdk5 may also contribute to the behavioral effects of cocaine [Patrick *et al.*, *Nature* 402:615-621 (1999); Nikolic *et al.*, *Genes Dev.* 10:816-825 (1996); Ohshima *et al.*, *Proc. Natl. Acad. Sci. USA* 93:11173-11178 (1996); Chae *et al.*, *Neuron* 18:29-42 (1997)].

Various publications are cited herein including those below, the disclosures of which are incorporated by reference in their entireties.

The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing

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description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are

5 approximate, and are provided for description.

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008707" 65678960

WHAT IS CLAIMED IS:

- 1 1. An isolated phosphorylated mammalian DARPP-32 protein comprising a
2 phosphorylated threonine residue; wherein the threonine residue can be reversibly
3 phosphorylated and dephosphorylated ; and wherein when the threonine residue is
4 dephosphorylated, it can be phosphorylated by Cdk5.
- 1 2. The phosphorylated mammalian DARPP-32 protein of Claim 1 which can
2 inhibit the kinase activity of cAMP-dependent protein kinase (PKA).
- 1 3. The phosphorylated mammalian DARPP-32 protein of Claim 2 wherein the
2 DARPP-32 protein has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:1
3 with a conservative amino acid substitution, and the threonine residue is the seventy-
4 fifth (75) amino acid residue of the amino acid sequence.
- 1 4. A phosphorylated fragment of a DARPP-32 protein, wherein the fragment of
2 the DARPP-32 protein comprises a phosphorylated threonine residue that when
3 dephosphorylated, can be phosphorylated by Cdk5.
- 1 5. The phosphorylated fragment of a DARPP-32 protein of Claim 4, wherein the
2 DARPP-32 protein has the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:1
3 with a conservative amino acid substitution, and the threonine residue is the seventy-
4 fifth (75) amino acid residue of the amino acid sequence.
- 1 6. A fusion peptide comprising the phosphorylated fragment of a DARPP-32
2 protein of Claim 4.
- 1 7. A chimeric protein comprising the phosphorylated mammalian DARPP-32
2 protein of Claim 1.

1 8. A phosphorylation state-specific antibody having specificity for
2 Thr75-phosphorylated DARPP-32 having the amino acid sequence of SEQ ID NO:1.

1 9. The antibody of Claim 8 which is a monoclonal antibody.

1 10. The antibody of Claim 9 which is a chimeric antibody.

1 11. A method of identifying an agent that can modulate the phosphorylation state of
2 Thr75 DARPP-32 comprising:

3 (a) contacting a potential agent with Cdk5 or an analog thereof, and
4 DARPP-32 or a Cdk5 phosphorylatable fragment of DARPP-32; and

5 (b) determining the amount and/or rate of phosphorylation of DARPP-32 or
6 the Cdk5 phosphorylatable fragment of DARPP-32; wherein the potential agent is
7 identified as an agent that can modulate the phosphorylation state of Thr75 DARPP-32
8 if the amount and/or rate of phosphorylation of DARPP-32 or the Cdk5
9 phosphorylatable fragment of DARPP-32 determined is significantly changed in the
10 presence of the potential agent relative to in its absence.

1 12. The method of Claim 11 further comprising:

2 (c) contacting the agent with an alternative protein kinase and a substrate
3 for that alternative kinase; wherein the alternative kinase is known not to
4 phosphorylate DARPP-32 on Threonine-75; and

5 (d) determining the amount and/or rate of phosphorylation of the substrate;
6 wherein the agent is identified as an agent that can modulate the phosphorylation state
7 of Thr75 DARPP-32 if the amount and/or rate of phosphorylation of the substrate is
8 not significantly changed in the presence of the agent relative to in its absence.

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1 13. The method of Claim 12 further comprising:

2 (e) administering the agent to a mouse along with a dopamine D1 receptor
3 agonist; wherein the administration of the dopamine D1 receptor agonist alone results
4 in an increase in the phosphorylation state of a cyclic-AMP dependent protein kinase
5 (PKA) substrate naturally occurring in the mouse; and
6 (f) determining the amount and/or rate of phosphorylation of the PKA
7 substrate; wherein the agent is identified when the amount and/or rate of
8 phosphorylation of the substrate is significantly decreased in the presence of the agent
9 relative to in its absence.

1 14. The method of Claim 13 wherein the agent can cross the blood brain barrier.

1 15. The method of Claim 14 further comprising:

2 (g) administering the agent to a DARPP-32 knockout mouse along with a
3 dopamine D1 receptor agonist; wherein the administration of the dopamine D1
4 receptor agonist alone results in an increase in the phosphorylation state of a cyclic-
5 AMP dependent protein kinase (PKA) substrate naturally occurring in the mouse; and
6 (h) determining the amount and/or rate of phosphorylation of the PKA
7 substrate; wherein the agent is identified when the amount and/or rate of
8 phosphorylation of the substrate is not significantly changed in the presence of the
9 agent relative to in its absence.

1 16. A method for treating dopamine dysregulation in an individual comprising
2 administering to the patient an agent that either inhibits the phosphorylation of
3 Thr75-DARPP-32 or promotes the dephosphorylation of Thr75-DARPP-32.

1 17. The method of Claim 16, wherein the dopamine dysregulation leads to a
2 symptom characteristic of a condition selected from the group consisting of
3 schizophrenia, Parkinson's Disease, Tourette's syndrome, Huntington's disease,
4 attention deficit hyperactivity and drug abuse.

- 1 18. The method of Claim 16, wherein the agent can cross the blood brain barrier.
- 1 19. The method of Claim 16 wherein the phosphorylation of Thr75-DARPP-32 is
2 inhibited by inhibiting Cdk5.
- 1 20. The method of Claim 19 wherein the agent is roscovitine.
- 1 21. The method of Claim 19 wherein the agent is a member of the class of
2 compound selected from the group consisting of an indirubin and a paullone.

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ABSTRACT OF THE INVENTION

The present invention discloses that DARPP-32 is substrate for the cyclin dependent kinase Cdk5. The phosphorylation takes place at a specific threonine residue of
5 DARPP-32 (Threonine 75). The Cdk5 catalyzed phosphorylation of DARPP-32 converts this protein into an inhibitor of the cAMP dependent protein kinase (PKA) and furthermore prevents it from being converted to an inhibitor of protein phosphatase 1 (PP1). Methods of identifying agents that modulate the phosphorylation of DARPP-32 by Cdk5 are disclosed. Methods of treating dopamine dysfunction in animal
10 subjects are also provided.

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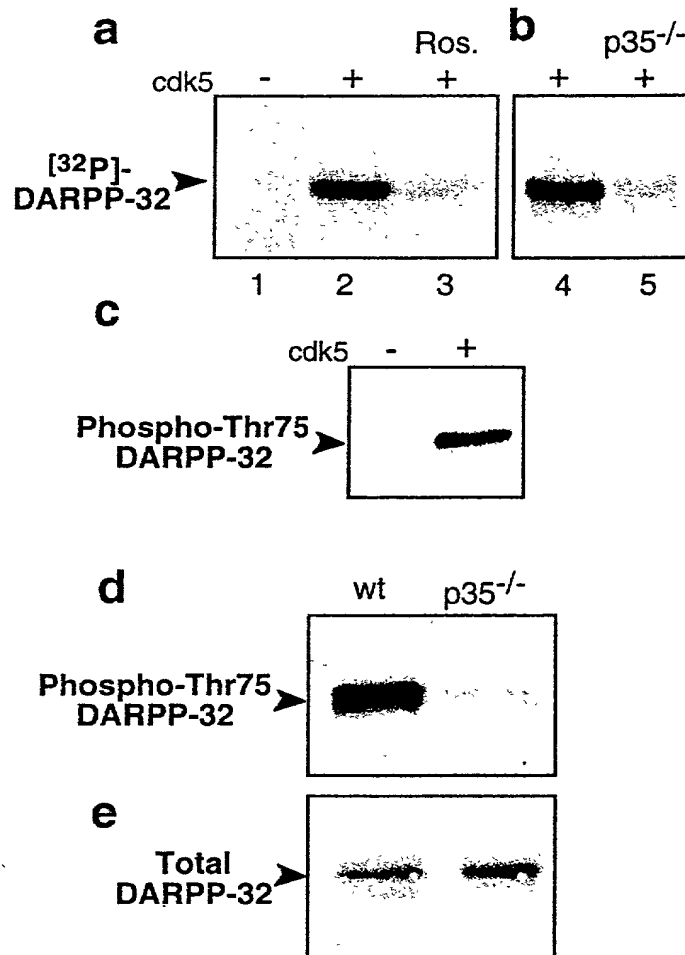


Figure 1

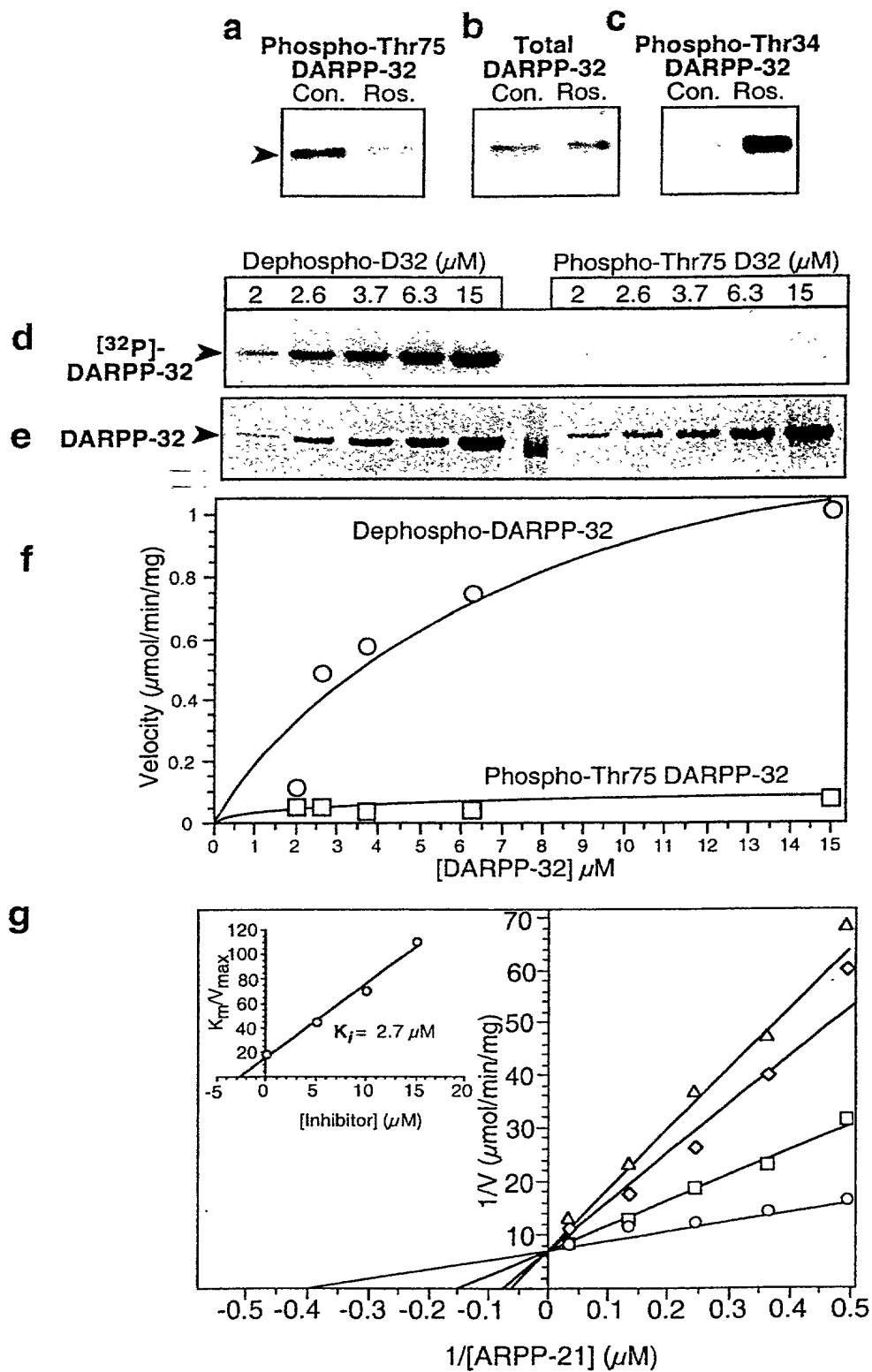


Figure 2

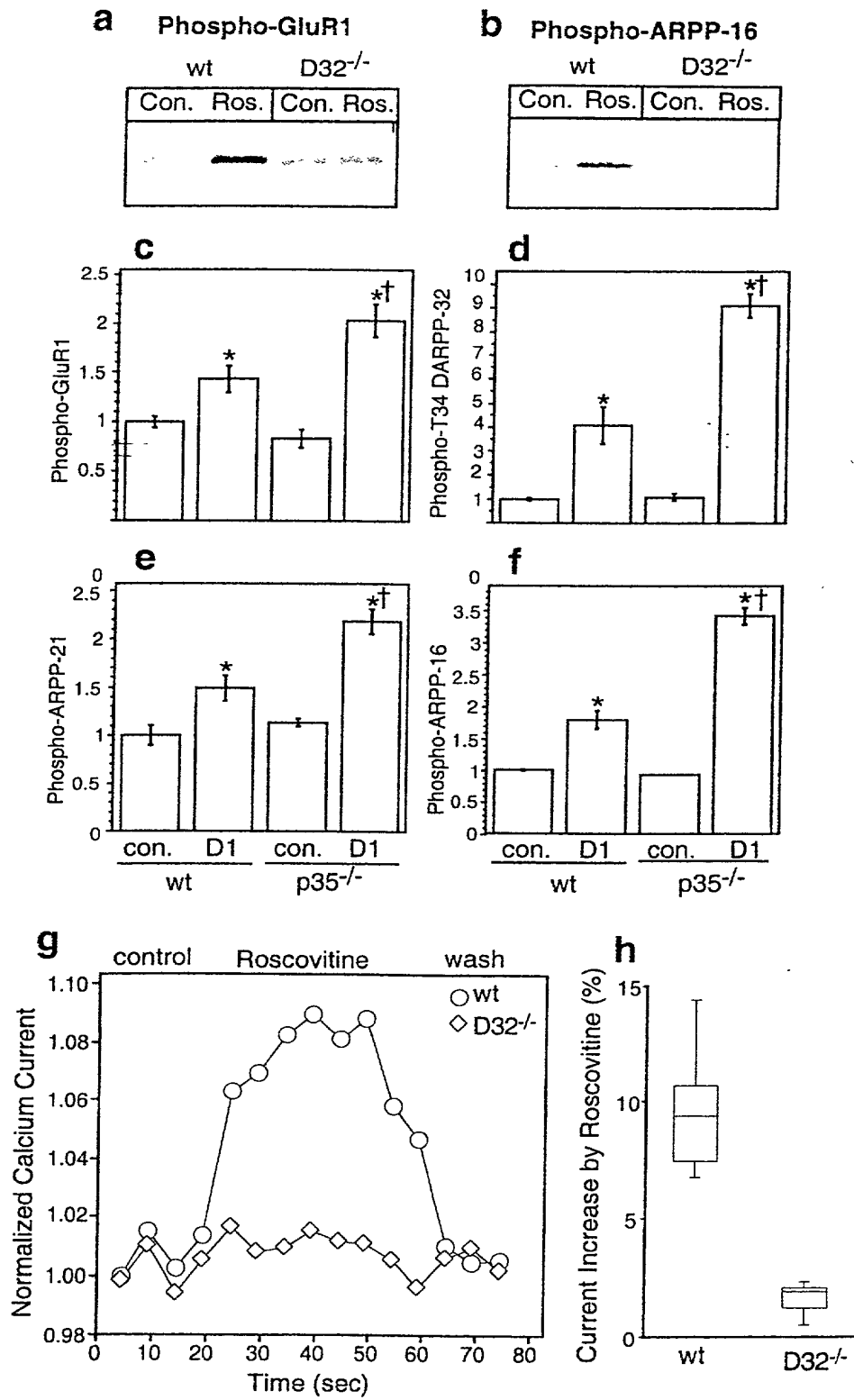


Figure 3

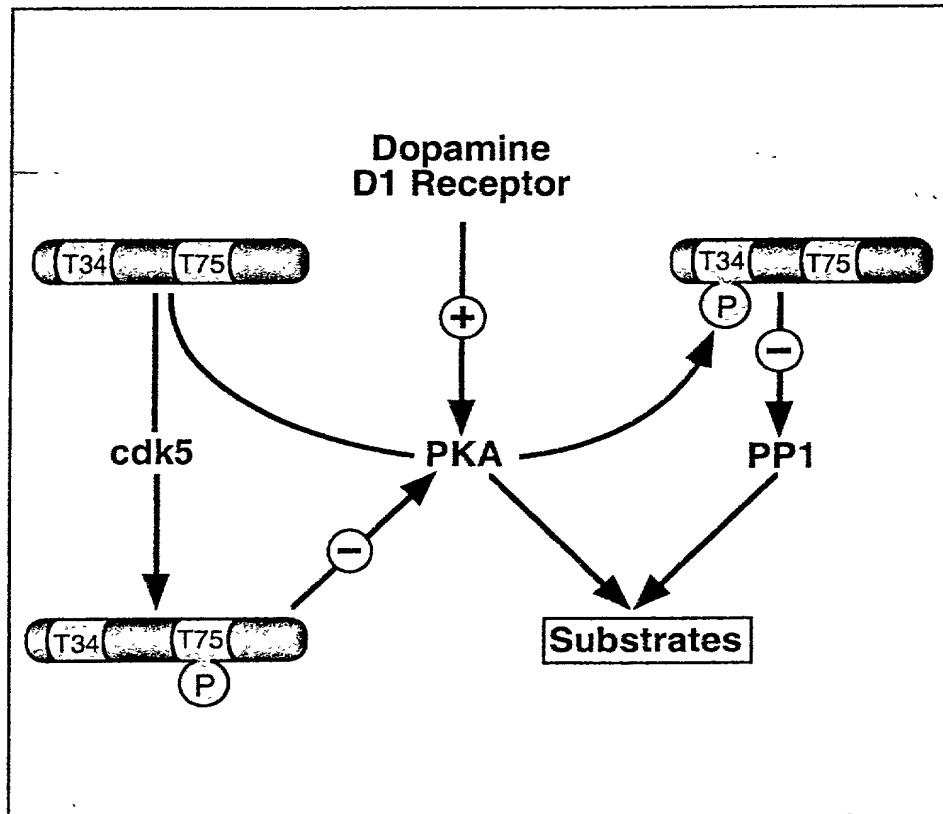


Figure 4A

Potential Therapeutic Targets

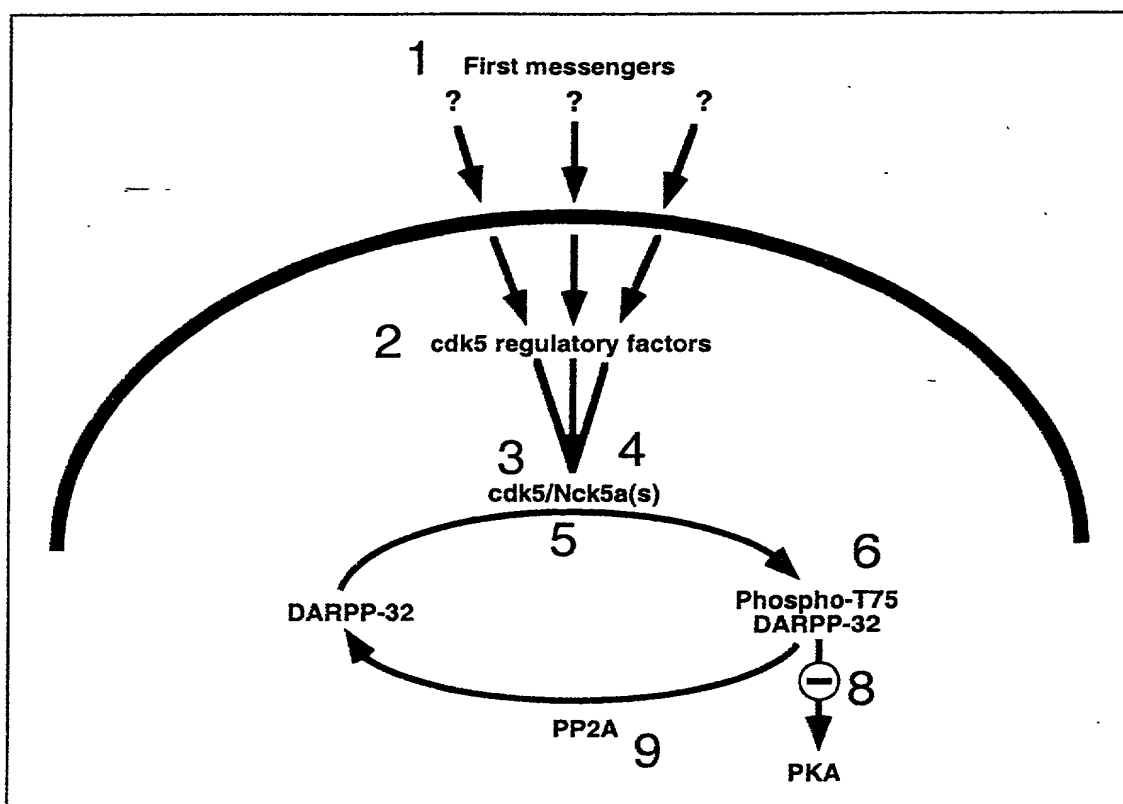


Figure 4B

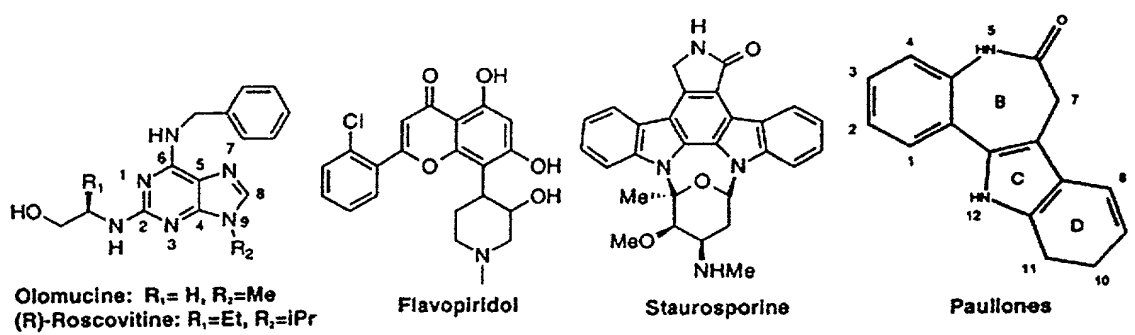
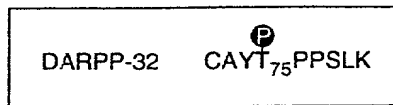
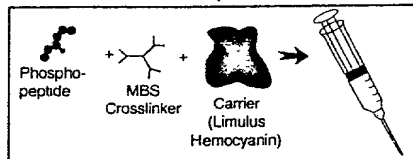


Figure 5

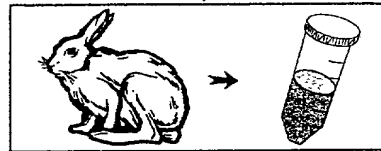
Production of Phosphorylation State-Specific Antibody

A

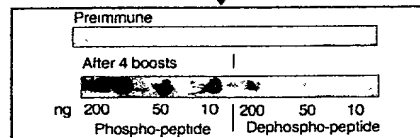
Following determination of the phosphorylated residue, a short synthetic phospho-peptide corresponding to the region surrounding the site is prepared.

B

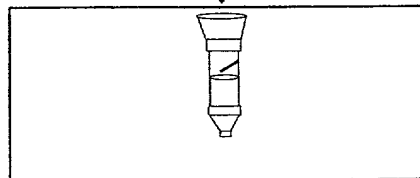
Phosphopeptides are conjugated to carrier protein and prepared for inoculation.

C

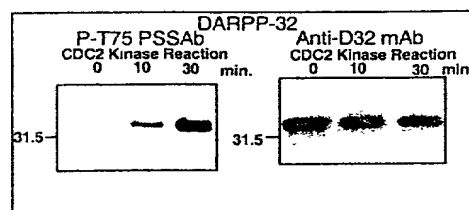
Inoculation, boosting, and bleeding of rabbits.

D

Primary screening of anti-sera by immuno-dot-blotting.

E

Purification of phospho-specific Ab using protein A and phospho/dephosphopeptide affinity chromatography.

F

Confirming specificity by immunoblotting

Steps involved in the generation of phosphorylation state-specific antibodies (PSSAb) are illustrated schematically. Typical results of phospho-peptide dot-blot screening of serum are shown. The bottom panel shows immunoblot results confirming the selectivity of the antibody for phosphorylated protein.

Figure 6

Figure 7

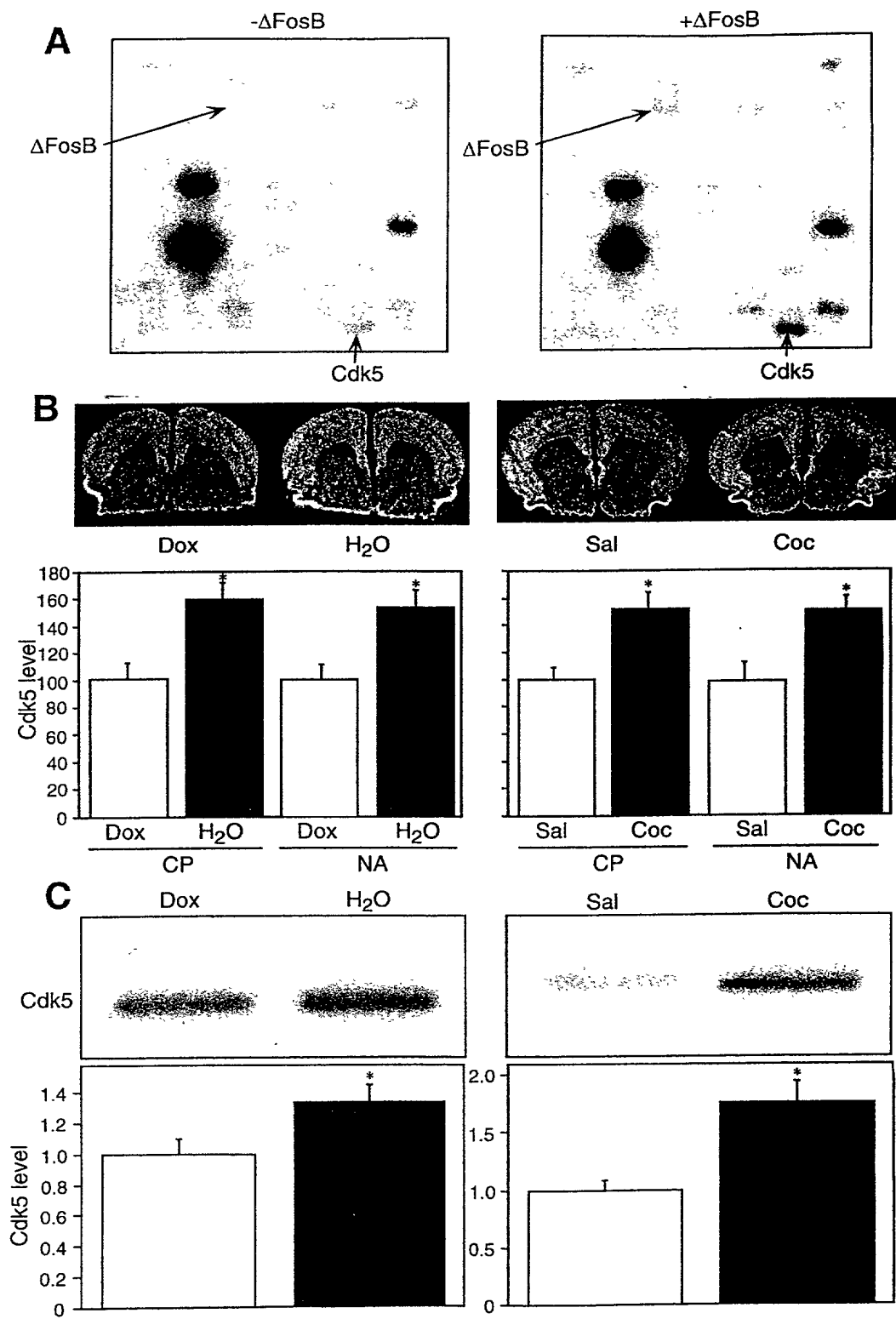


Figure 8

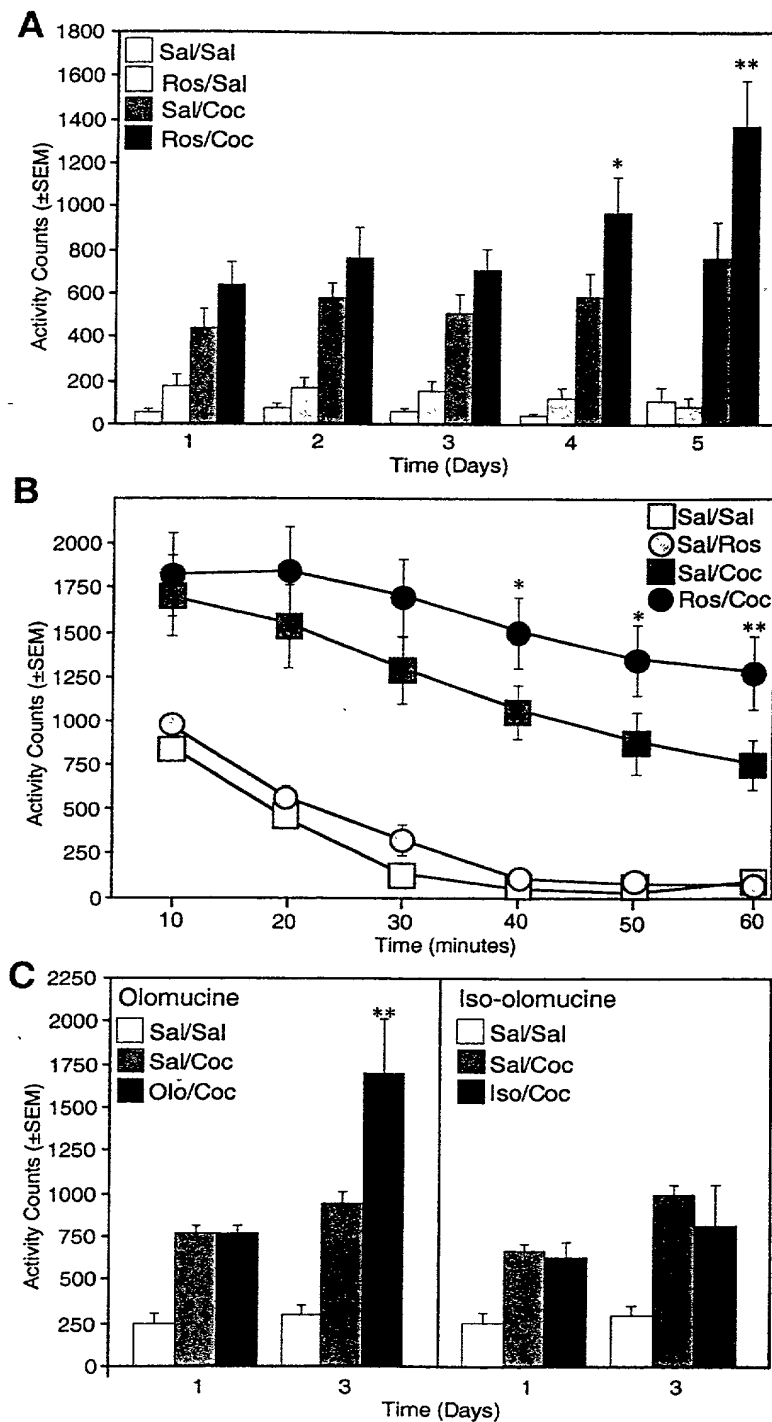


Figure 9

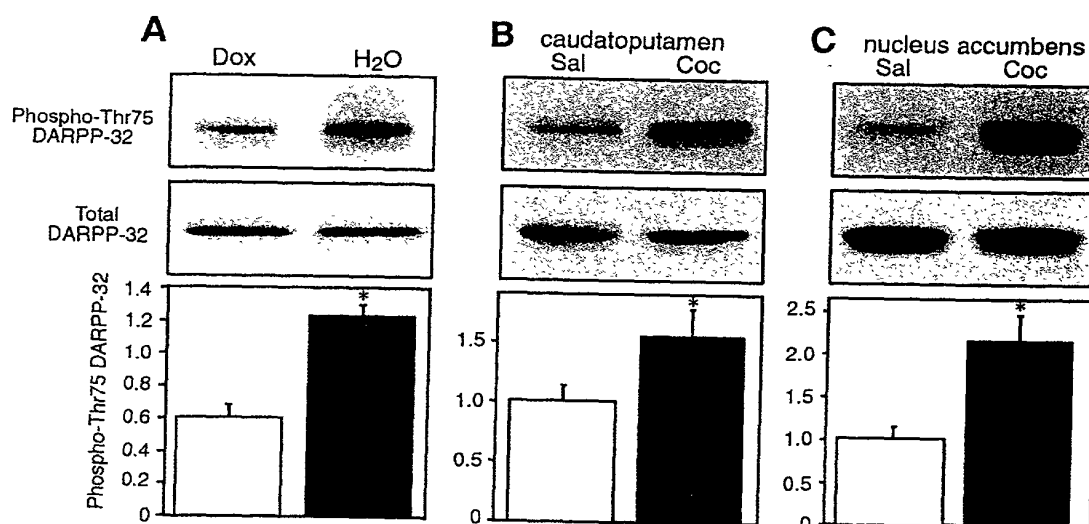


Figure 10

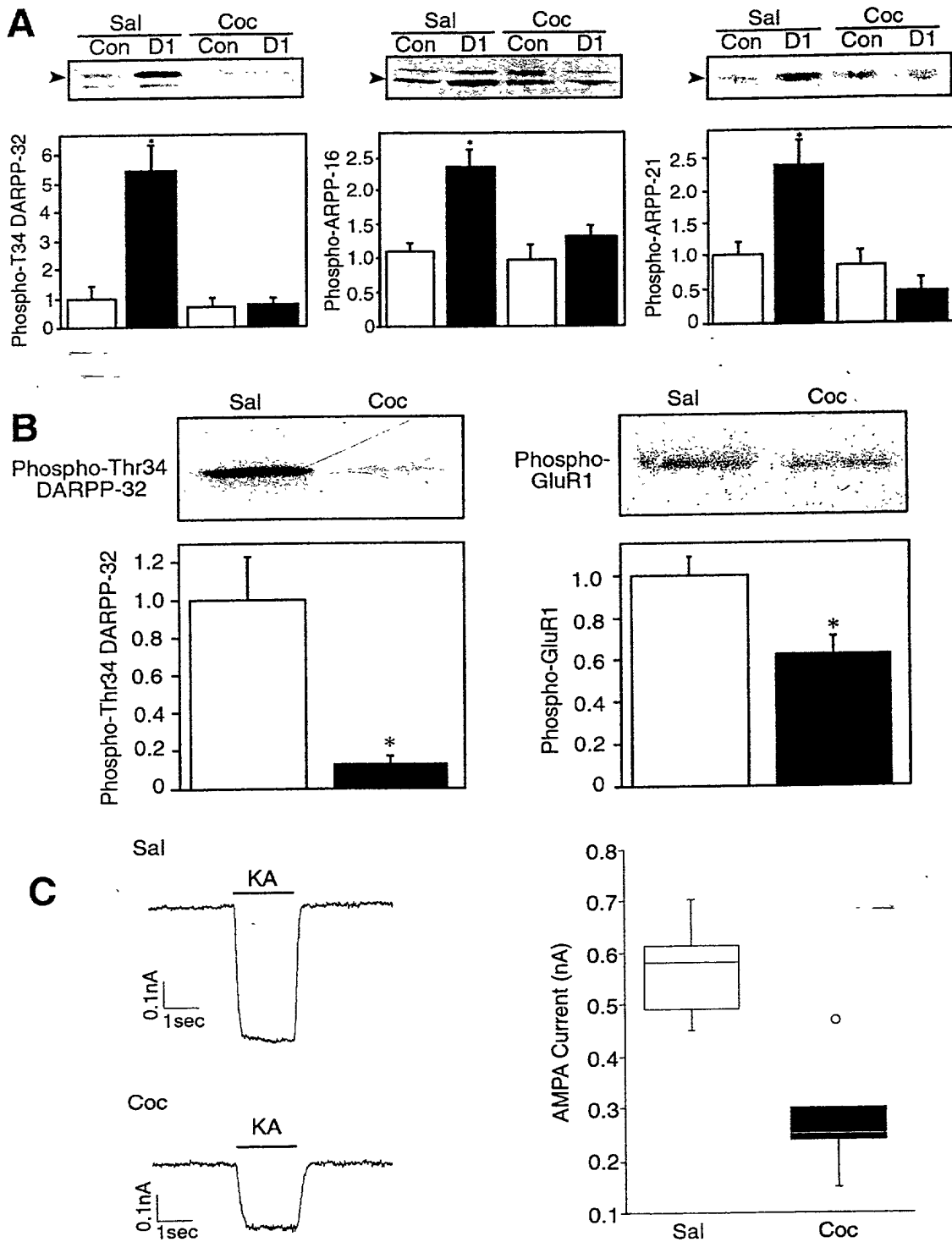


Figure 11

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As below named inventors, we hereby declare that:

Our residence, post office address and citizenship are as stated below under our names.

We believe that we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled

**METHODS OF IDENTIFYING AGENTS THAT REGULATE
PHOSPHORYLATION/DEPHOSPHORYLATION IN DOPAMINE SIGNALING**

the Specification of which

☒ is attached hereto
☐ was filed on _____
as Application Serial No. _____
and was amended on _____ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, §119 of any provisional application filed in the United States in accordance with 35 U.S.C. §1.119(e), or any application for patent that has been converted to a Provisional Application within one (1) year of its filing date, or any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

<u>PRIOR FILED APPLICATION(S)</u>			
<u>APPLICATION</u>	<u>COUNTRY</u>	<u>(DAY/MONTH/YEAR FILED)</u>	<u>PRIORITY</u>
<u>NUMBER</u>			<u>CLAIMED</u>

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in any prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a), which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION NO. _____	FILING DATE (DAY/MONTH/YEAR)	STATUS - PATENTED, PENDING, <u>ABANDONED</u>
09/419,379	15 OCTOBER 1999	PENDING

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from The Rockefeller University as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the person from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

I hereby appoint as my attorneys or agents the registered persons identified under

Customer No. 23565

for the law firm of Klauber & Jackson, said attorneys or agents with full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Please address all correspondence regarding this application to:

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411 HACKENSACK AVENUE
HACKENSACK, NEW JERSEY 07601

Direct all telephone calls to David A. Jackson at (201) 487-5800.

We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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DATE _____

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SIGNATURE OF INVENTOR _____

DATE _____

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<120> METHODS OF IDENTIFYING AGENTS THAT REGULATE
PHOSPHORYLATION/DEPHOSPHORYLATION IN DOPAMINE SIGNALING

<140> UNASSIGNED

<160> 4

<170> PatentIn Ver. 2.0

<210> 1

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Lys Ser Lys Arg Pro Asn Pro Cys Ala Tyr Thr Pro Pro Ser Leu Lys
65 70 75 80

Ala Val Gln Arg Ile Ala Glu Ser His Leu Gln Ser Ile Ser Asn Leu
85 90 95

Asn Glu Asn Gln Ala Ser Glu Glu Glu Asp Glu Leu Gly Glu Leu Arg
100 105 110

Glu Leu Gly Tyr Pro Arg Glu Glu Asp Glu Glu Glu Glu Glu Asp Asp
115 120 125

Glu Glu Glu Glu Glu Glu Glu Asp Ser Gln Ala Glu Val Leu Lys Val

Economic Indicators		Social Indicators		Environmental Indicators		Health Indicators		Education Indicators		Infrastructure Indicators	
Indicator	Value	Indicator	Value	Indicator	Value	Indicator	Value	Indicator	Value	Indicator	Value
GDP (USD)	123456789	Population (M)	123.45	Air Quality Index	123.45	Life Expectancy (Y)	78.90	Enrollment Rate (%)	98.76	Road Network (km)	123456
Unemployment (%)	5.67	Urbanization (%)	67.89	Water Quality Index	123.45	Infant Mortality (per 1000)	12.34	Graduation Rate (%)	95.67	Power Generation (MW)	12345
Inflation (%)	2.34	Gender Equality Index	123.45	Forest Cover (%)	12.34	Maternal Mortality (per 1000)	12.34	Research & Development (%)	12.34	Telecom Network (km)	123456
Trade Balance (USD)	123456789	Human Development Index	123.45	Renewable Energy (%)	12.34	Child Mortality (per 1000)	12.34	Healthcare Expenditure (%)	12.34	Internet Penetration (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Adult Literacy Rate (%)	98.76	Transportation Expenditure (%)	12.34	Mobile Phone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Water Recycling (%)	12.34	Primary Education Enrollment (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Secondary Education Enrollment (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Tertiary Education Enrollment (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Adult Literacy Rate (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Child Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Maternal Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Infant Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Adult Literacy Rate (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Primary Education Enrollment (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Secondary Education Enrollment (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Tertiary Education Enrollment (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Adult Literacy Rate (%)	98.76	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Child Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Maternal Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Infant Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
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FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste Recycling (%)	12.34	Child Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Inflow (USD)	123456789	Gender Inequality Index	123.45	Waste Recycling (%)	12.34	Maternal Mortality (per 1000)	12.34	Healthcare Infrastructure (%)	12.34	Smartphone Usage (%)	12.34
FDI Outflow (USD)	123456789	Gender Empowerment Measure	123.45	Waste							

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Pro Thr Pro Ala Leu Leu Phe Arg Val Ser Glu His Ser Ser Pro Glu
      35             40             45

Glu Glu Glu Glu Glu Ala Ser Pro His Gln Arg Thr Ser Gly Glu Gly
      50             55             60

His His Pro Lys Ser Lys Arg Pro Asn Pro Cys Ala Tyr Thr Pro Pro
      65             70             75             80

Ser Leu Lys Ala Val Arg Arg Leu Gln Thr Ile Ser Asn Leu Ser Glu
      85             90             95

Asn Gln Ala Ser Glu Glu Glu Asp Glu Leu Gly Glu Leu Arg Glu Leu
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Gly Tyr Pro Gln Glu Asp Asp Glu Glu Asp Glu Asp Glu Glu Glu Asp
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Glu Glu Glu Asp Ser Gln Ala Glu Val Leu Lys Gly Ser Arg Gly Thr
      130            135            140

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Val Gly Gln Lys Leu Leu Val Ala Gly Val Trp Arg Gly Pro Gly Ser
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Ala His Leu Leu Trp Met Ser Pro Arg Glu Met Glu Thr Leu Arg Thr
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Lys Trp Lys Ala Glu Gln His Glx Val Ser Leu Glu Arg Asn Leu Ser
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Ile Pro Ala Pro Pro Glu Pro Gly Thr
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<213> Rattus sp.

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Pro Thr Pro Ala Leu Leu Phe Arg Val Ser Glu His Ser Ser Pro Glu
35 40 45

Glu Glu Ser Ser Pro His Gln Arg Thr Ser Gly Glu Gly His His Pro
50 55 60

Lys Ser Lys Arg Pro Asn Pro Cys Ala Tyr Thr Pro Pro Ser Leu Lys
65 70 75 80

Ala Val Gln Arg Ile Ala Glu Ser His Leu Gln Thr Ile Ser Asn Leu
85 90 95

Ser Glu Asn Gln Ala Ser Glu Glu Glu Asp Glu Leu Gly Glu Leu Arg
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Glu Leu Gly Tyr Pro Gln Glu Asp Asp Glu Glu Asp Glu Asp Glu Asp
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Glu Glu Glu Asp Glu Glu Glu Asp Ser Gln Ala Glu Val Leu Lys Gly
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Ser Arg Gly Thr Ala Gly Gln Lys Leu Thr Ser Gly Gln Gly Leu Glu
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Gly Pro Trp Glu Arg Pro Pro Pro Leu Asp Glu Pro Gln Arg Asp Gly
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 <212> PRT
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 20 25 30

Pro Thr Pro Ala Met Leu Phe Arg Leu Ser Glu His Ser Ser Pro Glu
 35 40 45

Glu Glu Ala Ser Pro His Gln Arg Ala Ser Gly Glu Gly His His Leu
 50 55 60

Lys Ser Lys Arg Pro Asn Pro Cys Ala Tyr Thr Pro Pro Ser Leu Lys
 65 70 75 80

Ala Val Gln Arg Ile Ala Glu Ser His Leu Gln Ser Ile Ser Asn Leu
 85 90 95

Gly Glu Asn Gln Ala Ser Glu Glu Glu Asp Glu Leu Gly Glu Leu Arg
 100 105 110

Glu Leu Gly Tyr Pro Arg Glu Glu Glu Glu Glu Glu Glu Asp
 115 120 125

Glu Glu Glu Glu Glu Asp Ser Gln Ala Glu Val Leu Lys Gly Ser Arg
 130 135 140

Gly Ser Ala Gly Gln Lys Thr Thr Tyr Gly Gln Gly Leu Glu Gly Pro
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Trp Glu Arg Pro Pro Pro Leu Asp Gly Pro Gln Arg Asp Gly Ser Ser

09637959 101300

165

170

175

Glu Asp Gln Val Glu Asp Pro Ala Leu Asn Glu Pro Gly Glu Glu Pro
180 185 190

Gln Arg Met Pro Ala His Pro Glu Pro Gly Thr
195 200